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REACTIONS OF THE CONNECTIVE TISSUE IN CHICKENS TO *PLASMODIUM GALLINACEUM* AND *PLASMODIUM LOPHURAE*

I. HISTOPATHOLOGY DURING INITIAL INFECTIONS AND SUPERINFECTIONS

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This study is based on tissues taken from 589 chickens initially infected or superinfected with either *Plasmodium gallinaceum* or *Plasmodium lophurae*. The experiments differed with respect to the age of the chickens; the amount and, hence, virulence of the infecting and the superinfecting inoculums; and the kind of parasite, i.e., sporozoite or blood stage, injected. In presenting the histopathological findings, special attention has been paid to the cellular changes connected with innate and acquired immunity. They include, on the positive side, such things as phagocytosis, the development of new macrophages, lymphoid hyperplasia and various reparative processes, and, on the negative side, the destruction of phagocytes, lymphocytes and red cells.

The phagocytic aspects, such as the relation of phagocytosis to immunity and the production of new macrophages, are similar to the earlier studies of Cannon and Taliaferro (1931), Taliaferro and Cannon (1936), Taliaferro and Mulligan (1937) and Singer (1954). They will, therefore, be presented briefly with special references to differences and in sufficient detail to supply a basis for the biochemical studies in Part II (Moulder and Taliaferro, 1955). The interplay between lymphocytopoiesis and lymphoid depletion in the production of various connective tissue cells involved in immunity and repair will be considered in greater detail because it carries further previous studies by Conway (1938 and 1939) and Taliaferro and Pizzi (1955). The findings are of particular interest because of the intense pathogenicity of *P. gallinaceum* and the great reactivity of avian connective tissue (Taliaferro and Bloom, 1945).

The earliest reference to pathological changes in infections of *P. gallinaceum* was made by Brumpt (1936). Three recent studies have appeared. One is a preliminary report on *P. gallinaceum* by Hershberger and Coatney (1946), and the other two are on *P. lophurae* by Terzian (1941) and Rigdon (1944). The pathological processes involved in various malarias and the cellular basis for malarial immunity have been reviewed by Taliaferro and Mulligan (1937), Taliaferro (1941) and Maegraith (1948).

A review of the cellular basis of immunity by Taliaferro (1949) gives the authors' concepts of the classification, relationships and developmental potencies of the connective tissue cells involved. For convenience in the present paper, we have designated as monocytoid lymphocytes those cells intermedi-

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ate in structure between lymphocytes and monocytes and as medium polyblasts those inflammatory macrophages intermediate in size and structure between agranulocytes and larger macrophages. We have used lymphoid cell to include all lymphocytes, hemocytoblasts, various basophil stem cells of the different myeloid series and plasma cells.

MATERIALS AND METHODS

The origin and maintenance of the malarial species have been previously described by us (1945). The chickens were chiefly white leghorns or white legrocks (leghorn-plymouth rock hybrids). All were obtained as 2-day old chicks and reared in the laboratory. The normal mean weight increased with age as shown in table 1, but the chickens lost weight during intense malaria. Both normal and infected animals were kept on an alternate 12-hour light and dark schedule with the light period beginning at 8 a.m. Within the period of 6 years during which the tissues were collected, the colony was remarkably free of bird epizootics.

Parasite counts per 10 red cells were made from Giemsa-stained dried peripheral blood films according to the method described by W. H. and L. G. Taliaferro (1940). Blood smears were made at daily or 2-day intervals through the early developed infection and at weekly intervals thereafter.

Nodules from a given spleen were counted in 20 low power microscopic fields using a ×10 ocular and ×10 objective (22.6 sq mm) and are recorded in figures 2, 3, 4, 6 and 7 per microscopic low power field (1.13 sq mm). Active macrophages and their pigment content in text figure 5 are depicted per oil immersion field (×10 ocular and ×90 objective) which measured 0.014 sq mm. The sections were 10 μ thick.

Small pieces of tissues were taken from freshly sacrificed chickens and from a few chickens that had just died. Those collected for study consisted of the spleen, liver, bone marrow (from the femur near the epiphysis), bursa Fabricii, lung, brain, thymus, kidney, heart, breast muscle, intestine, pancreas and gonad. All were fixed in Zenker-formol (Helly-Maximow) from 4 to 6 hours, and the lung before being removed was injected in situ with fixative. The tissues were then embedded in nitrocellulose and, after sectioning, were stained with Maximow's hematoxylin-eosin azure II, as described by Buchsbaum and Loosli (1936). They were usually sectioned at 10 μ, but some spleens were cut at 5 μ to study to better advantage the development of lymphocytes through intermediate polyblasts to macrophages and the phagocytic activity of macrophages.

TABLE 1.—*Mean weight of normal chickens from 2 days to 24 weeks of age.*

Age in weeks	Weight in kg
0	0.04
2	0.08
4	0.17
8	0.49
12	0.80
16	1.00
24	1.50

EXPERIMENTAL RESULTS

Initial infection with P. gallinaceum

The histopathological picture of initial infection with *P. gallinaceum* was studied in 10 series of chickens as outlined in table 2. The series consisted of 165 small (<0.45 kg) and 219 large (>0.45 kg) chickens infected with either blood parasites or sporozoites.* Varying numbers of parasites were given per chicken initially at the rate of 10^6 to 10^9 blood parasites or 1/50th to 5 infected mosquitoes. These amounts resulted in (1) nonlethal infections (cf. text fig. 1E) especially in large chickens with the peak of the infection usually varying from 1 to 10 parasites per 10 red cells, (2) sublethal infections in the small chickens of series 2, 3 and 8, which were characterized by high peaks up to 22 parasites per 10 red cells (multiply infected red cells) and an intense parasitemia for a week or more with subsequent recovery (text fig. 1F to H), or (3) lethal infections in the chickens of series 1, 5 and 7 to the extent that most of the chickens died in 4.5 to 9 days and only a few survived (fig. 1A to D). The parasitemias in blood- and sporozoite-induced infections varied in the same range as regards the acute rise of the patent infection,

* Dr. Helen Jordan kindly prepared the sporozoites.

TABLE 2.—*Chickens initially infected with* P. gallinaceum. *One to six normal chickens were killed at intervals in each series.*

Series	Number of chickens	Approximate weight in kg when injected	Infection*				Used in	
			Parasite peak per 10 red blood cells	Day of peak	Type	When killed	Plate	Text figure
				A. Blood-induced				
1	29	0.4	12–16	4– 6	Lethal	1 hour–9 days†	1:5 2:1, 2, 3 4:1–3 5:2 6:1 9:1, 5	1, 2
2	32	0.4	20	11	Persisting	5–27 days		1, 2
3	15	0.2	15–20		Developed	17–37 days		1, 2
4	38	0.5	1–12		Developed	1– 7 months	2:4	1
5	28	1.0	17–22	4	Lethal	2–12 days		1, 2
6	47	1.5	1–14	5	Nonlethal‡	2–18 days	9:3, 4	1, 2
				B. Sporozoite-induced				
7	38	0.2	5–13	9	Lethal	1–14 days	1:3 7:2 8 9:2	1, 2
8	51	0.2	3–10	9–10	Sublethal	2–40 days	1:1, 2, 4 2:5 3:1–5 5:1 6:2, 3 7:1	1, 2
9	66	0.9	2–9	10	Nonlethal	9–24 days		1, 2
10	40	0.5	0.4–12	8–10	Developed	0.5– 8 months		1, 2

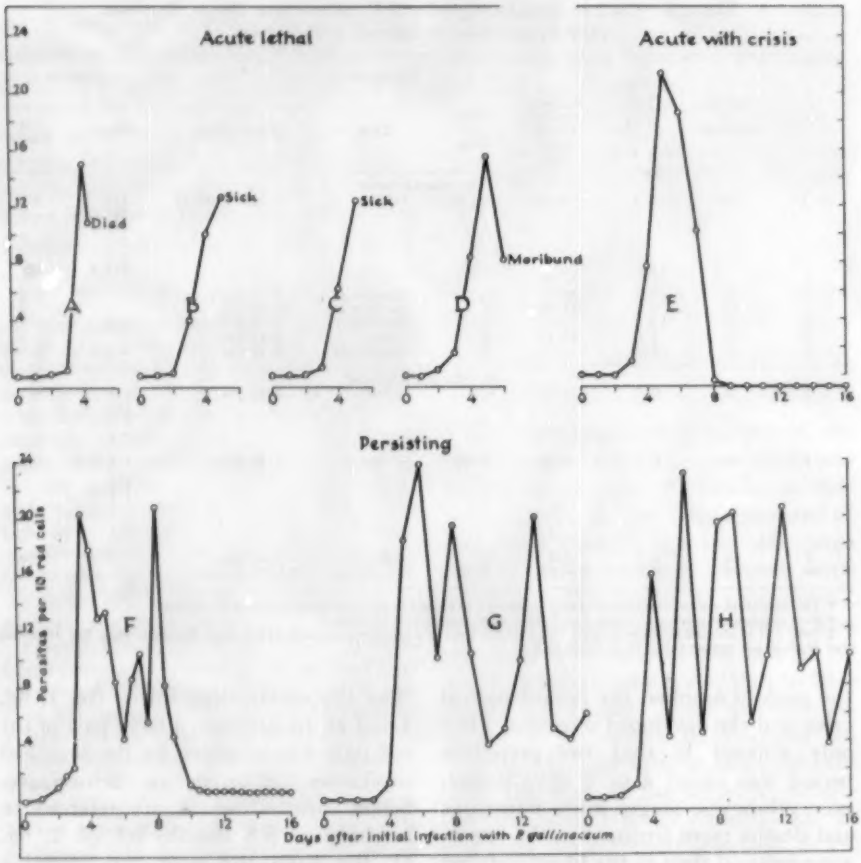
* The prepatent period varied from 1 day in series 1 to 6 days in the sporozoite-induced infections.
† Five chickens surviving in series 1 at 9 days were transferred to series 2.
‡ Only 2 of 49 chickens died in series 6. Only spleen, liver and bone marrow were taken from these chickens. See *Materials and Methods* for tissues taken from other series.

the peak in number, the parasitological crisis and the developed infection. They only differed in that the prepatent period was about 4 or 5 days longer, exoerythrocytic stages more numerous and deaths more frequent in the sporozoite-induced than in the blood-induced infections.

Hypertrophy of the spleen and liver as contrasted to other organs, such as the lung, kidney and heart, occurred during infection as is described in Part II of this series (Moulder and Taliaferro, 1955), which deals with the glucose metabolism of tissue slices.

Spleen

Uninfected chickens.—In our material, the spleen of the chicken, as in the canary (Cannon and Taliaferro, 1931) and other birds (Whiting 1894–96), contained normally less trabecular scaffolding and nodular lymphatic tissue than the mammalian spleen (pl. 1, fig. 1 and 2). In addition, a large part of the red pulp was occupied by the sheathed capillaries (ellipsoids or Schweigger-Seidel sheaths) which are referred to hereafter as S-S sheaths (cf. pl. 2, fig. 1). We have also used the following terminology: (1) red pulp or loose lymphatic tissue and (2) white pulp consisting of (a) dense non-nodular accumulations of lymphocytes within the stroma, mainly around the arteries and rarely around the S-S sheaths, which for convenience are designated as periarterial and perisheath accumulations, and (b) nodular lymphatic tissue generally surrounded by dense non-nodular lymphatic tissue. Perisheath and periarterial dense lymphatic accumulations were meager in amount. When present (pl. 1, fig. 2), they involved more small lymphocytes and were less extensive than in the malaria-induced infiltra-



TEXT FIG. 1.—Typical parasitemia curves of blood-induced *P. gallinaceum* infections in small chickens: A to B, lethal; E to H, intense but nonlethal. There is a longer prepatent period in sporozite-induced infections.

tions. Several small chickens among the controls showed a lymphoid hyperplasia comparable to the minimal hyperplasia found during malaria. These had probably suffered some kind of infection or other pathological process.

Nodules were scarce in the majority of chickens. They usually ranged in size from small (<0.002 sq mm) to medium (approximately 0.003 to 0.01 sq mm). In general, they contained varying mixtures of small, medium and large lymphocytes (pl. 4, fig. 2). Some consisted mainly of small ones (pl. 4, fig. 1) and an occasional one was markedly depleted (pl. 4, fig. 3) in that many reticular cells were visible. The lymphocytes were fairly active as evidenced by the fact that 2 or more mitotic figures were found in over 90% of the nodules. Frequently, mitoses occurred around the edges of the nodules where large lymphocytes predominated. It was an interesting finding that more medium- than small-sized nodules occurred in chickens weighing from 200 to 400 g than in large chickens. They were also more active. Thus, 42% of 55 nodules

studied in 12 small chickens had from 5 to 24 divisions per nodule, whereas only 3% of 50 nodules in 10 large chickens had more than 5 divisions per nodule ($P<0.001$ when the data were subjected to the χ^2 test).

Besides the ordinary tissue lymphocytes classified as small, medium and large, mention should be made of the very large lymphocyte (pl. 5, large lymphocyte G), which occurred to some extent in normal chickens and frequently during malaria. As compared to the ordinary large lymphocyte (pl. 4, fig. 2) it (1) was larger, (2) had more cytoplasm which stained as basophil as the cytoplasm of the plasma cell and exhibited clear vacuoles or clefts, and (3) had a nucleus which contained smaller chromatin granules placed on a delicate linin network and one or two larger nucleoli. This large lymphocyte was described in malaria by Taliaferro and Mulligan (1937) as a giant large lymphocyte and has been frequently described in infections or immune processes under several names. These include large lymphocyte, lymphoblastic plasma cell, macrohistiocyte, myeloblast, acute splenic tumor cell, basophil macrophage and developing or immature plasma cell (see review by Taliaferro, 1949). Observations presented in this paper suggest that it has at times all of the developmental potencies suggested by the foregoing names.

P. gallinaceum: initial infection.—Changes in the spleen roughly paralleled the course of the infection and varied with the length of the prepatent period and intensity of the infection. Exoerythrocytic (E-E) stages were first seen in the S-S sheaths as noted by Huff (1954) and were found more frequently there than in the red pulp.

During the acute rise of the infection, phagocytosis by the macrophages increased. It was first seen as soon as parasites appeared in the peripheral blood and consisted of one to several pigment granules in macrophages in the S-S sheaths (cf. Kyes, 1916). These active macrophages were either the fixed cells of the actual sheath or free macrophages originally present in or recently migrated between the cells of the sheath (cf. later stage in pl. 6, fig. 1). Early in the infection, activity by the macrophages in the red pulp surpassed that in the S-S sheaths. This supremacy was maintained throughout the remainder of the infection (cf. text fig. 5). On the 3rd to the 5th day, macrophages in the red pulp containing identifiable parasitized red cells reached a peak in numbers. They also sporadically contained recognizable remains of lymphocytes and granulocytes. The heavier the infections were, the more numerous were the swollen engorged macrophages.

Apparently normal uninfected red cells were constantly phagocytosed during the late acute rise, peak, crisis and immediately thereafter in mild infections (cf. Taliaferro and Cannon, 1936; Zuckerman, 1945). Often macrophages were distended with as many as 16 red cells (either parasitized or unparasitized). (Exact counts were impossible because of overcrowding.) Probably the same thing occurred in lethal infections but was masked by large amounts of previously phagocytosed pigment. It is a curious finding that the nuclei of engulfed red cells lost their stain sooner than the cytoplasm during the process of macrophage digestion. Macrophages in the S-S sheaths only rarely contained identifiable debris except for pigment clumps.

Beginning on the 6th day, identifiable parasitized red cells decreased in number in individual macrophages as digestion proceeded, while clumps of pigment accumulated. At this time, the red pulp recalled the inflammatory-like foci described by Taliaferro and Pizzi

(1955). In intense infections, many overengorged macrophages with their burden of relatively indigestible pigment clumps became cyst-like and their nuclei failed to stain normally. Large numbers of them died and disintegrated. Thereafter, vestiges of them frequently persisted for a month, but were eventually taken up by other macrophages. Such an extensive destruction of macrophages was not noted by Taliaferro and Mulligan (1937) in the acutely fatal infections of *Plasmodium knowlesi* in rhesus monkeys.

The foregoing sequence took place after a delay of about 4 days in sporozoite-induced infections. This length of time represented the longer prepatent period.

Phagocytosed pigment clumps were always more or less evenly distributed during the acute rise throughout the red pulp, but they began to coalesce in the macrophages as early as the 8th day, and the engorged macrophages tended to aggregate at the edges of the red pulp after the crisis. In some chickens, at 3 weeks, irregular blocks of pigment at the edges of the red pulp or in swirls around the nodules made an arresting histological picture. They were frequently surrounded by large lymphocytes and plasma cells. (This redistribution was not as pronounced in persisting or long-continued low grade patent infections because of the continuing phagocytic activity of more or less unengorged macrophages in the red pulp.) Relatively enormous isolated aggregations of pigment sometimes measured 35 μ in cross section at 3 months. They gradually disappeared, but small masses of pigment were sometimes still present along with mildly active macrophages at 6 months.

As the parasitemia rose, various intermediate stages between lymphocytes and macrophages occurred. These were not found in uninfected chickens. They were best seen in splenic veins or sinuses although a complete series was seldom found in a small area. Thinly cut well-stained sections were an advantage. We interpret the common occurrence of these intermediate cells during infection in conjunction with the frequent mitotic proliferation of lymphocytes (see later) and the rarely seen mitoses in reticular cells as indicating the heteroplastic cytogenesis of macrophages from lymphocytes and consider the process one of the chief functional aspects of lymphoid hyperplasia. As most workers agree that the latter part of this process occurs, i.e., the development of monocytes into macrophages, only the early stages are depicted in plate 9, figures 3 and 4. The development involved a swelling of the cytoplasm with a loss of basophil staining when large lymphocytes were involved and a lightening of the staining of the nucleus as it became more vesicular. Intermediate polyblasts containing parasitized erythrocytes and particularly pigment could be found which still retained nuclei characteristics of lymphocytes.

Lymphoid depletion was closely followed by lymphoid hyperplasia but these two activities did not proceed equally in all chickens or even in a given series similar with respect to age, dose and/or kind of parasite injected. Thus, on the 5th day, some chickens in series 1 showed an over-all marked depletion, others a marked hyperplasia. Concomitantly, many nodules in an individual chicken were depleted: others were not. In one chicken in series 5 on the 8th day, depletion occurred in the S-S sheaths but not in the periarterial accumulations.

Lymphoid depletion of all areas of the spleen often overshadowed lymphoid proliferation early in the acute rise. Frequently, just before or during the crisis of lethal infections, almost all of the lymphocytes disappeared from the hy-

perplastic non-nodular tissue surrounding the S-S sheaths (pl. 1, fig. 3). In sick or moribund chickens, atypical, dead or dying cells, especially lymphocytes and engorged macrophages, occurred throughout the spleen. Not all chickens died at peak parasitemia. They also died during the crisis and early developed infection when lymphoid hyperplasia was well started. Similar findings were reported for monkeys infected with *Plasmodium brasilianum* (Taliaferro and Cannon, 1936).

Lymphocyte hyperplasia was apparent as a great excess of dense lymphatic tissue. It was associated with a mobilization of very large lymphocytes from reticular cells (pl. 9, fig. 2) and an increase in dividing lymphocytes. Mitosis, especially of the large and medium lymphocytes, was most prevalent in the nodules, intermediate in the rest of the white pulp and least frequent in the red pulp. In this last mentioned site, a dividing lymphocyte was sometimes seen in every one or two oil immersion fields. Nodules frequently contained 10 to 20 mitoses: at times even more. Clumps of 3 or 4 dividing large lymphocytes were often encountered at the edge of a nodule. The very large lymphocyte became increasingly numerous in all of the lymphatic tissue, particularly at the periphery of nodules and of non-nodular white pulp. Associated with this intense lymphocytic proliferation, some but relatively few reticular cells divided.

When lymphocyte replacement reached the stage of a definite lymphoid hyperplasia, increased numbers of lymphocytes, especially of the medium and large sizes, occurred in the red pulp, in larger areas of perisheath and periarterial dense accumulations and in many more and larger than normal nodules (pl. 3, fig. 1). In addition, numerous small focal accumulations of the very large lymphocytes were localized between the non-nodular accumulations and red pulp or at the edge of nodules. They eventually changed into large plasma cells and, in turn, became typical Marschalkó plasma cells as their over-all and nuclear size decreased (pl. 7, fig. 1). As previously mentioned, these nests of large lymphocytes and plasma cells sometimes surrounded masses of redistributed pigment.

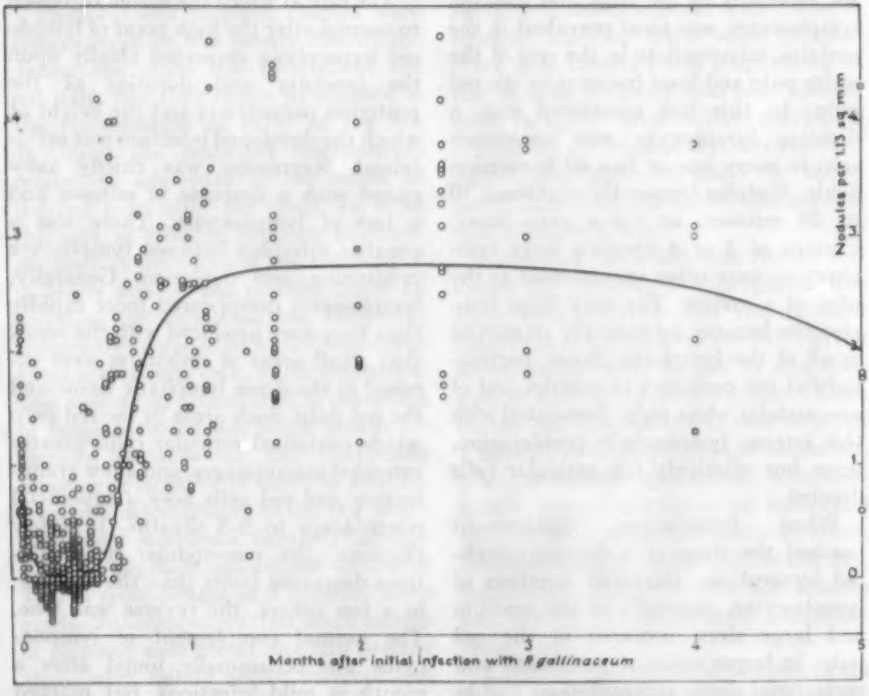
In general, intense mitotic proliferation of lymphocytes was associated with a lymphocytocidal activity and considerable lymphorrhexis. At such times, various lymphocytic remnants were seen in macrophages. Affected lymphocytes had pycnotic nuclei, and many exhibited abnormal and contracted mitotic figures.

The rate at which the spleen regressed to normal after the high point of lymphoid hyperplasia depended chiefly upon the intensity and duration of the postcrisis parasitemia and the height at which the developed infection was maintained. Regression was chiefly associated with a decrease in mitoses and a loss of lymphocytes. There was a peculiar interplay between lymphocyte production and depletion. Generally, lymphocytes disappeared more rapidly than they were produced with the result that small areas of reticulum were exposed in the dense lymphatic tissue and the red pulp. Such areas in the red pulp which contained reticular cells, greatly extended macrophages, and a few granulocytes and red cells bore a superficial resemblance to S-S sheaths. In a few chickens, the non-nodular accumulations decreased faster than the nodules; in a few others, the reverse was true. The normal complement of lymphocytes was occasionally found after a month in mild infections, but marked hyperplasia sometimes persisted for 6 months.

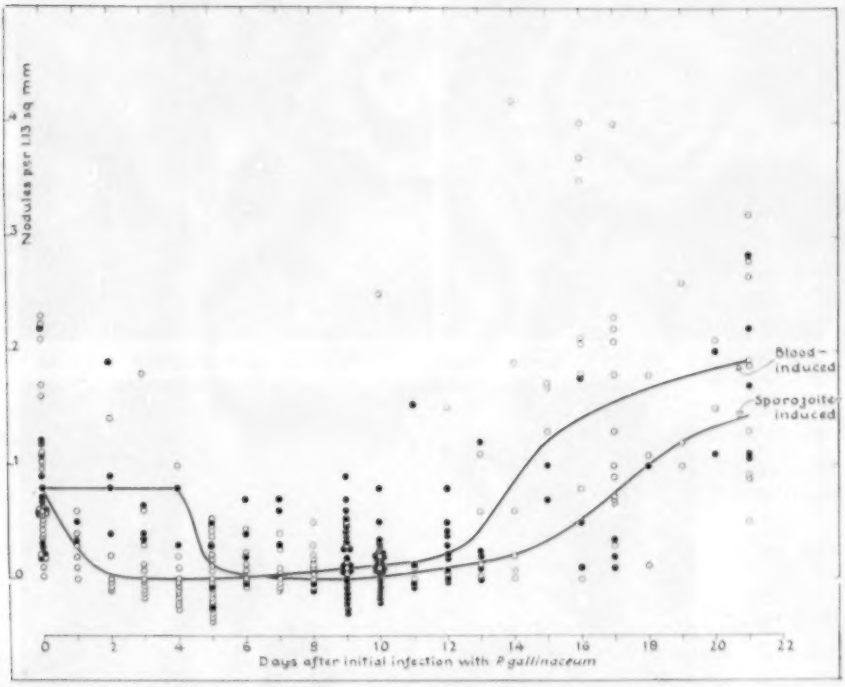
The number of nodules in the spleen constituted an excellent index of the

over-all degree and extent of lymphoid depletion and hyperplasia and parallels the impressions gained from careful general studies of the lymphoid changes. Nodule counts per 1.13 sq mm are given in text figure 2 from 38 normal chickens and from 354 chickens representative of all series in table 1 during infection with *P. gallinaceum* for 5 months. The data, especially as illustrated by the visually fitted curve, conclusively demonstrate a pronounced loss of nodules associated with an initial lymphoid depletion during the 1st week, recovery and beginning regeneration during the 2nd and 3rd weeks, over-regeneration at 1 to 3 months and regression after 3 months. The range, however, was wide for any given day due to the variability in lymphoid activities. Thus, the count varied from 0.05 to 2.3 nodules in normal chickens although it was usually from 0.3 to 1.2 nodules. (Six chickens uninfected with malaria which contained 1.6 to 2.3 nodules were probably activated by some condition other than malaria.) It also varied from 0.8 to 3.2 nodules a month after infection and from 1.6 to 4.7 nodules 2.5 months after infection. One chicken, which contained the largest amount of pigment seen in a spleen, still showed depletion (0.2 nodule) at 40 days and two chickens still showed over-regeneration (3.8 and 4.3 nodules) at 6 months.

The nodule counts were further separated in text figure 3 for the first 21 days of the infection with respect to



TEXT FIG. 2.—Nodules per 1.13 sq mm in the spleen for 5 months after initial infection with *P. gallinaceum*. Note their initial disappearance, reappearance and abnormally large number during the 1st through 3rd month and gradual regression thereafter. Each circle represents data from one chicken and the curve was visually fitted from mean values. The same procedure was used in text figures 3, 4, 6 and 7.

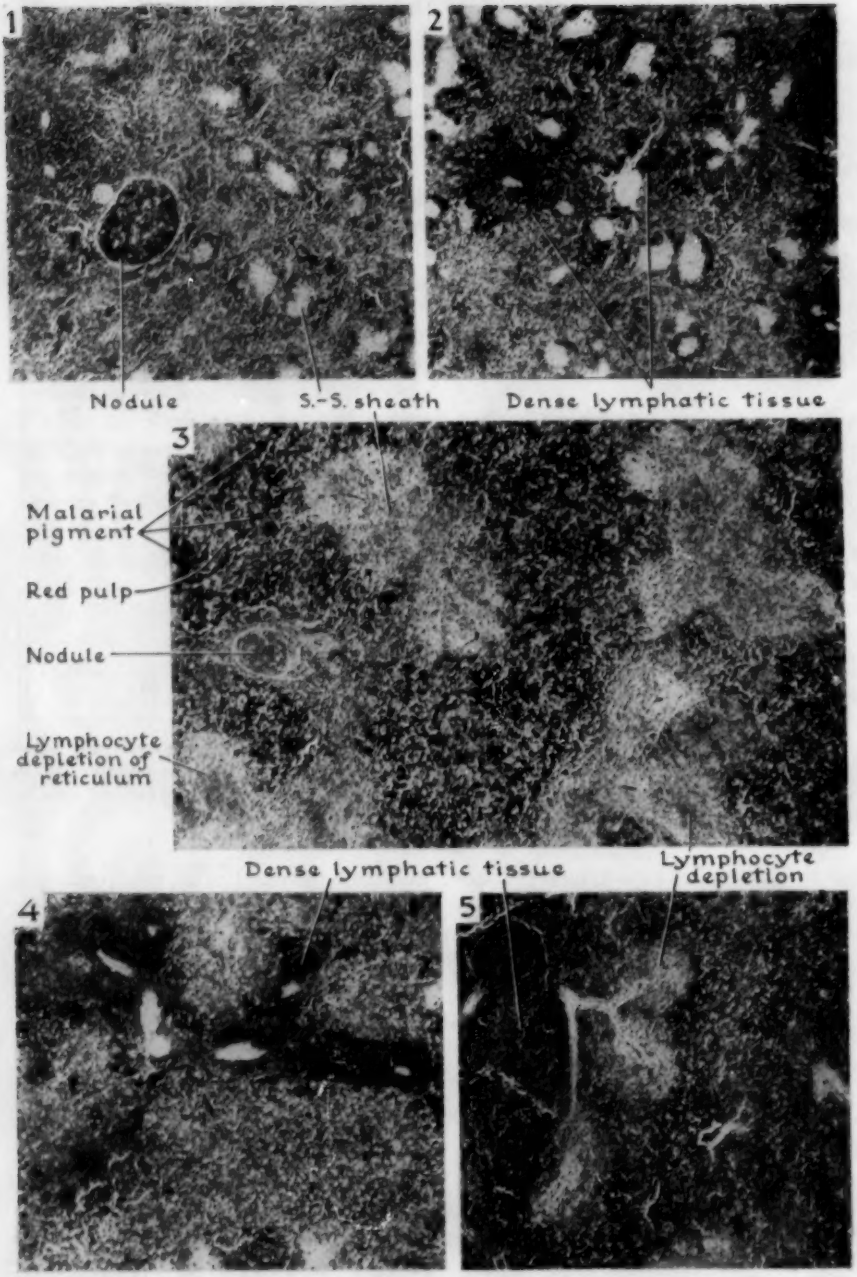


TEXT FIG. 3.—Nodules per 1.13 sq mm in the spleen for 21 days after initial infection with blood parasites (open circles) and sporozoites (solid circles) of *P. gallinaceum*. Note the reflection of the longer prepatent period in sporozoite-induced infections as compared to the blood-induced ones by the delayed disappearance and reappearance of nodules.

blood- and sporozoite-induced infections. Here the time after infection is magnified 6 times as compared to figure 2 to depict the early part of the infection more clearly. In general, nodules decreased markedly one day after blood-induced infection, were virtually absent on the 3rd, 4th and 5th days and remained scarce for 7 more days. From the 14th through the 17th days, some chickens made a remarkable recovery while others did not. For example, on the 14th day, the count was 0 for one chicken and 4.2 nodules for another. The functional difference between two such spleens is remarkable. In contrast, nodules did not decrease until the 5th day in sporozoite-induced infections and reached their lowest value on the 9th and 10th days. The longer prepatent period and the later peak in parasitemia in the sporozoite-induced infections as compared to the blood-induced infections were thus clearly paralleled by a correspondingly later lymphoid depletion. A delay was also found during recovery.

Erythropoiesis occurred as early as 4 days after infection and was encountered sporadically during the patent parsitemia. It was often associated with lymphoid infiltration of the red pulp. The very large lymphocytes apparently served as hemocytoblasts and could be connected to the polychromatophil proerythroblasts by a continuous series of intermediate forms (pl. 6, fig. 3).

Granulocytes were sometimes less



Legends for Plates

All photomicrographs and drawings are from tissues of chickens either normal or infected with *P. gallinaceum* (plates 1 through 9) or *P. lophurae* (plate 10). The drawings were made with the aid of a

frequent in the red pulp during the early acute rise (around 10 per 0.014 sq mm) and more frequent during the late acute rise, peak and crisis (up to 30 to 80 per 0.014 sq mm). There were normally around 20 per 0.014 sq. mm. They may have mobilized as a result of the inflammatoid reactions associated with the marked destruction of parasites and host cells. During this phase and for a week or so after the initiation of the developed infection, extramedullary myelopoiesis was found. It subsided gradually. During the developed and latent infection, granulocytes varied from normal to above normal numbers.

Most of the statements so far have described the events during lethal and persisting infections. Reactions in mild infections as in series 6 and 9 were more stereotyped. They resembled the changes characteristic of lophurae infections (cf. text fig. 5 and 6). Thus, phagocytic and lymphoid activities were surprisingly uniform in the spleens of 16 chickens from series 9 which were killed on the 9th day even though parasitemias varied from 0.2 to 1 per 10 red cells. The same may be said of 16 chickens from the same series killed on the following day with parasitemias varying from 1.5 to 4.1 per 10 cells. Marked differences, however, occurred between the two groups. On the 10th day, phagocytosis of parasitized cells had greatly increased and depletion had markedly advanced.

From the foregoing account, it may be concluded that following an initial marked lymphoid depletion in non-lethal infections, the production of lymphocytes (and other lymphoid cells) overshadowed and counterbalanced the loss of lymphocytes incident to infection and supplied a mesenchymal reserve for the heteroplastic development of macrophages, red cells, plasma cells and, to a less extent, granulocytes. In lethal infections, the production of lymphocytes did not counterblance depletion.

Liver

Uninfected chickens.—The normal liver was mainly free of periportal lymphoid accumulations, but occasionally contained small lymphatic nodules. Some of the nodules were associated with large portal vessels. Others located without any relation to the central or portal veins may have developed from earlier foci of local inflammation. Several animals were not considered within the normal baseline because the accumulations of lymphoid cells were associated with extramedullary myelopoiesis

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camera lucida. Details of the infections may be found in tables 2 through 4 and the histological techniques in the section on *Materials and Methods*.

PLATE 1.—Photomicrographs of normal spleens (fig. 1 and 2) and of lymphoid depletion and hyperplasia in spleens after infection of *P. gallinaceum* (fig. 3–5). ×80.

PL. 1, FIG. 1—Normal spleen (series 8) showing a typical lymphatic nodule and the virtual absence of perisheath lymphoid accumulations.

PL. 1, FIG. 2—Normal spleen (series 8) showing the upper normal limit of lymphoid accumulations around the Schweigger-Seidel sheaths.

PL. 1, FIG. 3—Marked lymphoid depletion of non-nodular tissue around the Schweigger-Seidel sheaths during the beginning crisis from an 8-day lethal infection (series 7). Note that the red pulp contains most of the malarial pigment and is not depleted of lymphocytes.

PL. 1, FIG. 4.—Partial return of perisheath lymphocytes in the depleted stroma and the development of areas of non-nodular dense lymphatic tissue during the postcrisis from a 14-day infection (series 8).

PL. 1, FIG. 5.—Marked increase in the non-nodular dense perisheath lymphatic tissue from a 4.5-day terminal infection (series 1) with some lymphocyte depletion.

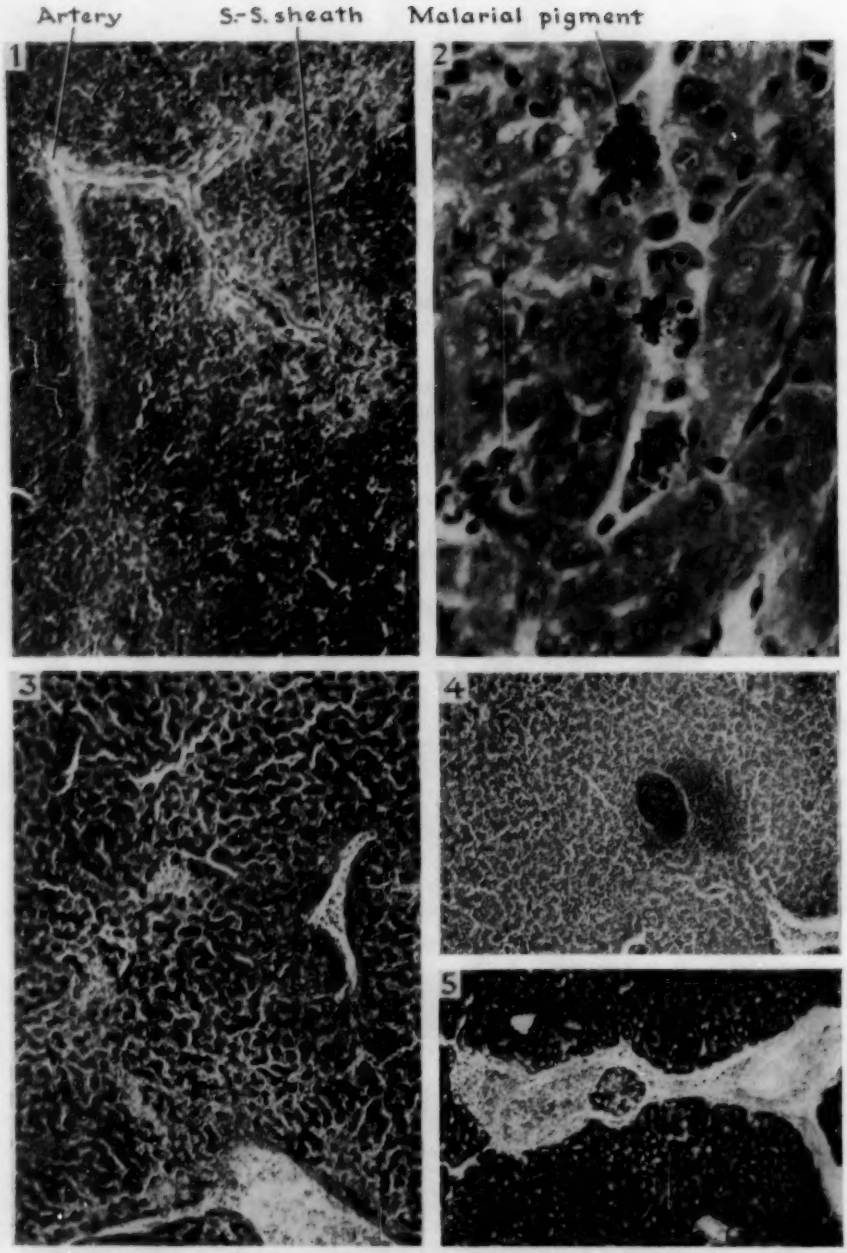


PLATE 2.—Photomicrographs of miscellaneous changes associated with terminal infections (fig. 1 to 3 and 5) or persisting infections (fig. 4) of *P. gallinaceum*.

PL. 2, FIG. 1.—Pigmented macrophages in the Schweigger-Seidel sheaths of the spleen from a 4.5 day terminal infection (series 1). Note the relationship between the arterioles and the surrounding Schweigger-Seidel sheaths. This is a magnification of part of the section shown in plate 1, figure 5. ×122.

PL. 2, FIG. 2.—The accumulation of pigment in Kupffer cells of the liver from a 6-day infection (series 1). ×760.

PL. 2, FIG. 3.—Disorientation of the liver cords from a 9-day terminal infection (series 1). × 80.

PL. 2, FIG. 4.—Nodule in the periportal connective tissue of the liver from a 57-day infection (series 4). ×80.

PL. 2, FIG. 5.—Nodule in the pancreas from a 9-day sublethal infection (series 8). ×80.

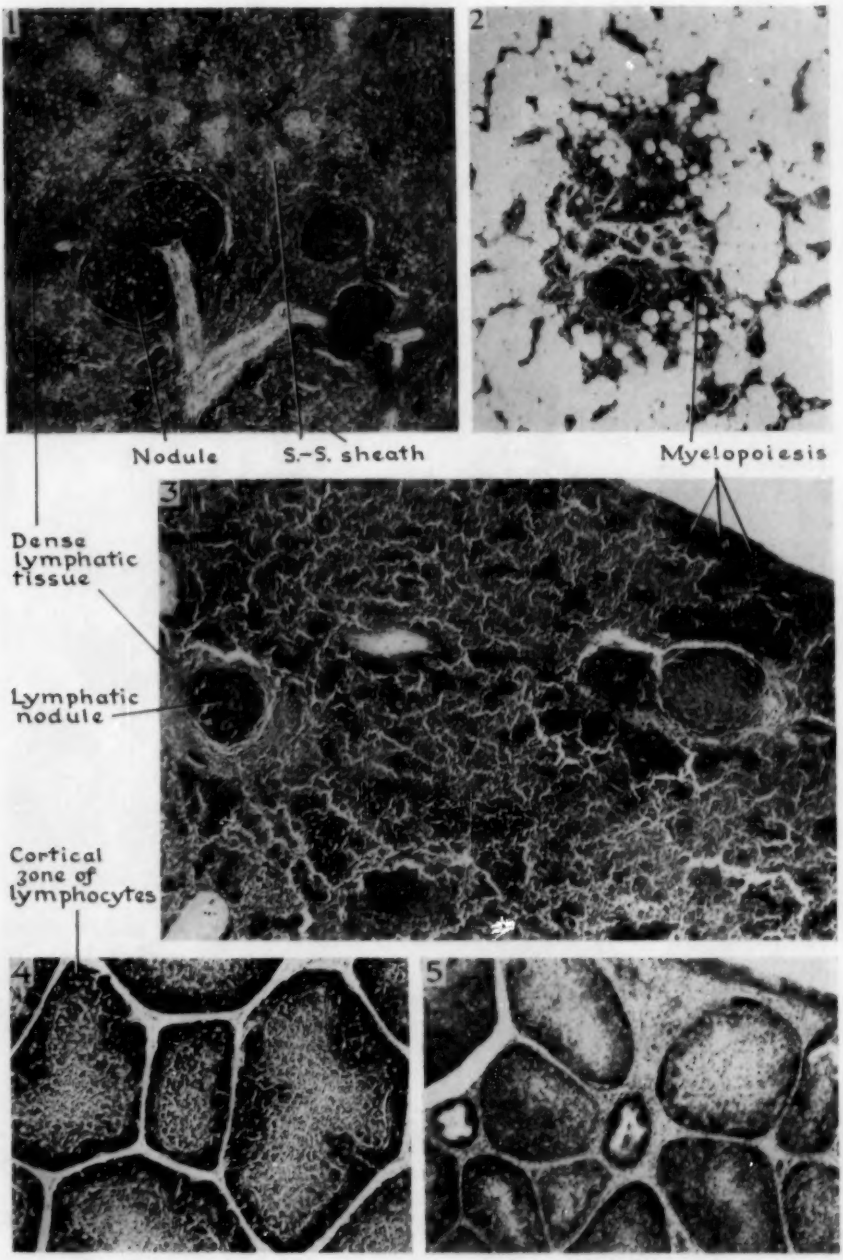


PLATE 3.—Photomicrographs of normal tissues (fig. 2 and 4) and of miscellaneous changes in tissues during persisting infections of *P. gallinaceum* (fig. 1, 3 and 5). ×80.

PL. 3, FIG. 1.—Spleen from a 16-day infection (series 8) showing an increase in nodular and non-nodular dense lymphatic tissue and a replacement of lymphocytes around the S-S sheaths.

PL. 3, FIG. 2.—Normal bone marrow showing a lymphoid nodule in the myeloid tissue (series 8).

PL. 3, FIG. 3.—Hyperplastic bone marrow from a 14-day infection (series 8) showing widespread granulopoiesis, erythropoiesis within the sinusoids, and several lymphatic nodules surrounded by dense lymphatic tissue.

PL. 3, FIG. 4.—Normal bursa Fabricii (series 8).

PL. 3, FIG. 5.—Bursa Fabricii from a 12-day infection (series 8) showing a marked loss of lympho cytes.

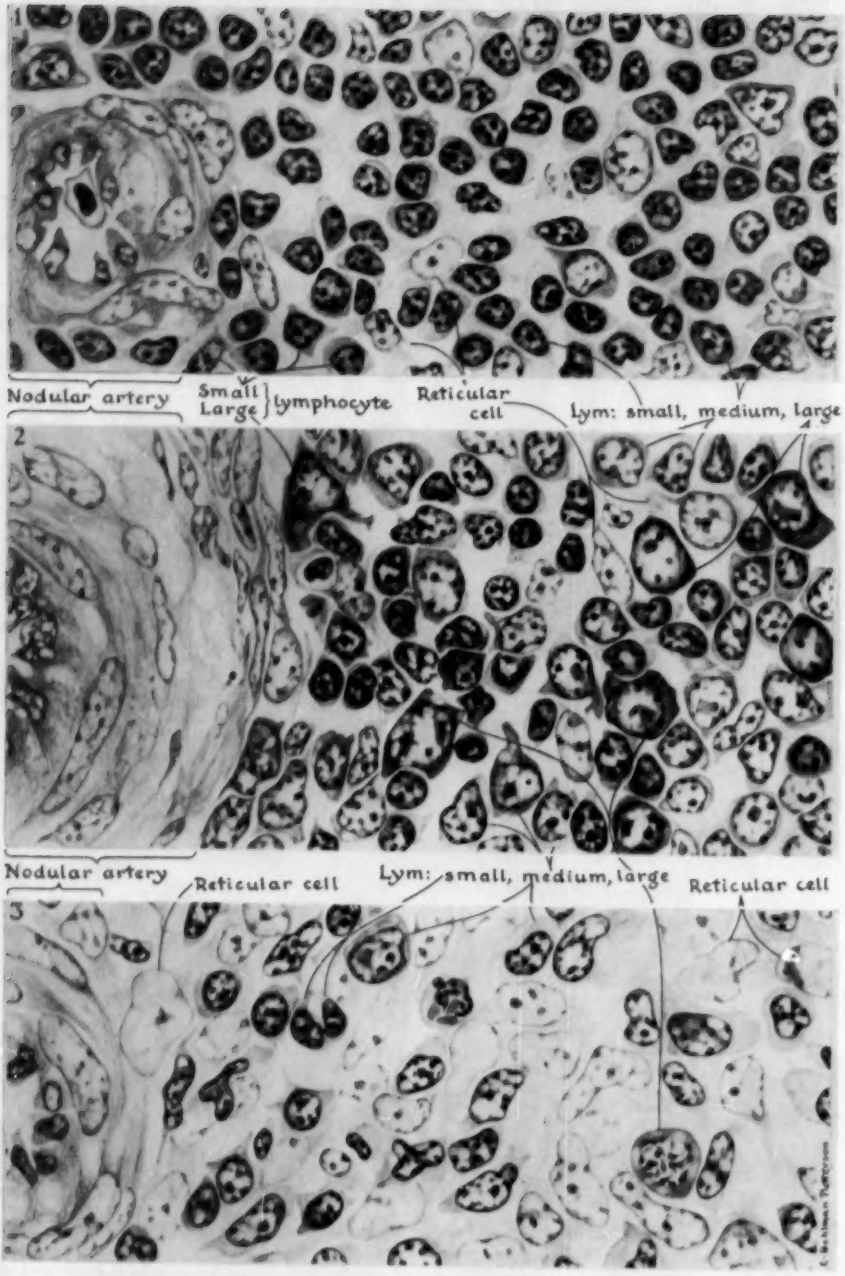


PLATE 4.—Drawings of three types of nodules in normal spleens (series 1). ×1900.

PL. 4, FIG. 1.—Nodule consisting predominantly of small lymphocytes which extend inward to the nodular artery.

PL. 4, FIG. 2.—Nodule containing many medium and large lymphocytes. Transitions between this type of nodule and the one in the preceding figure often occur.

PL. 4, FIG. 3.—Nodule showing marked lymphocyte depletion and the exposure of reticular cells of the stroma.

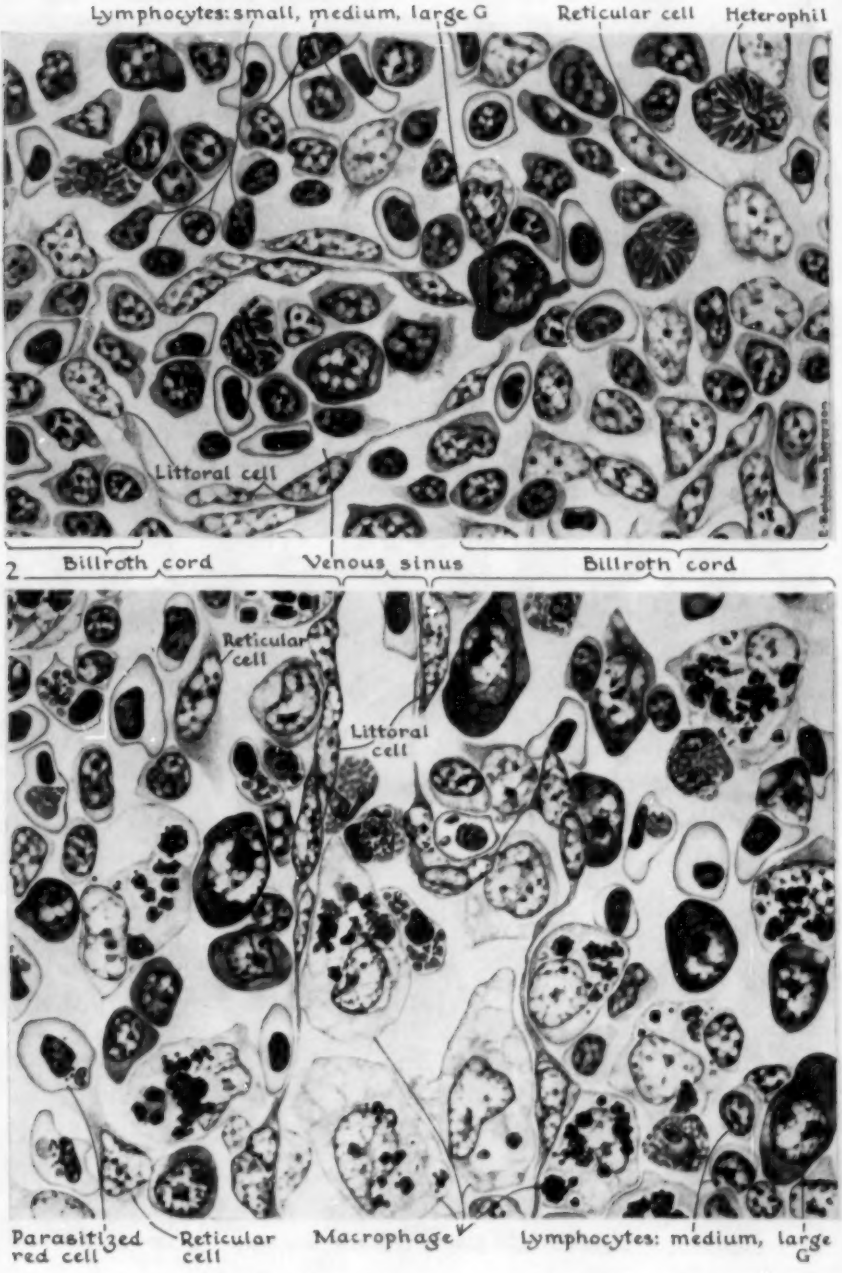


PLATE 5.—Drawings from the red pulp of the spleen before and after infection with *P. gallinaceum* (series 1). ×1900.

PL. 5, FIG. 1.—Lymphocytes of all sizes including large basophil ones in both the cords and sinuses of a normal spleen.

PL. 5, FIG. 2.—Engorged macrophages in the cords and especially in the sinuses from a terminal infection (5.2-day infection). Note that some of the macrophages contain whole parasitized red cells as well as malarial pigment and that there is a reversal in the normal ratio of small and large lymphocytes.

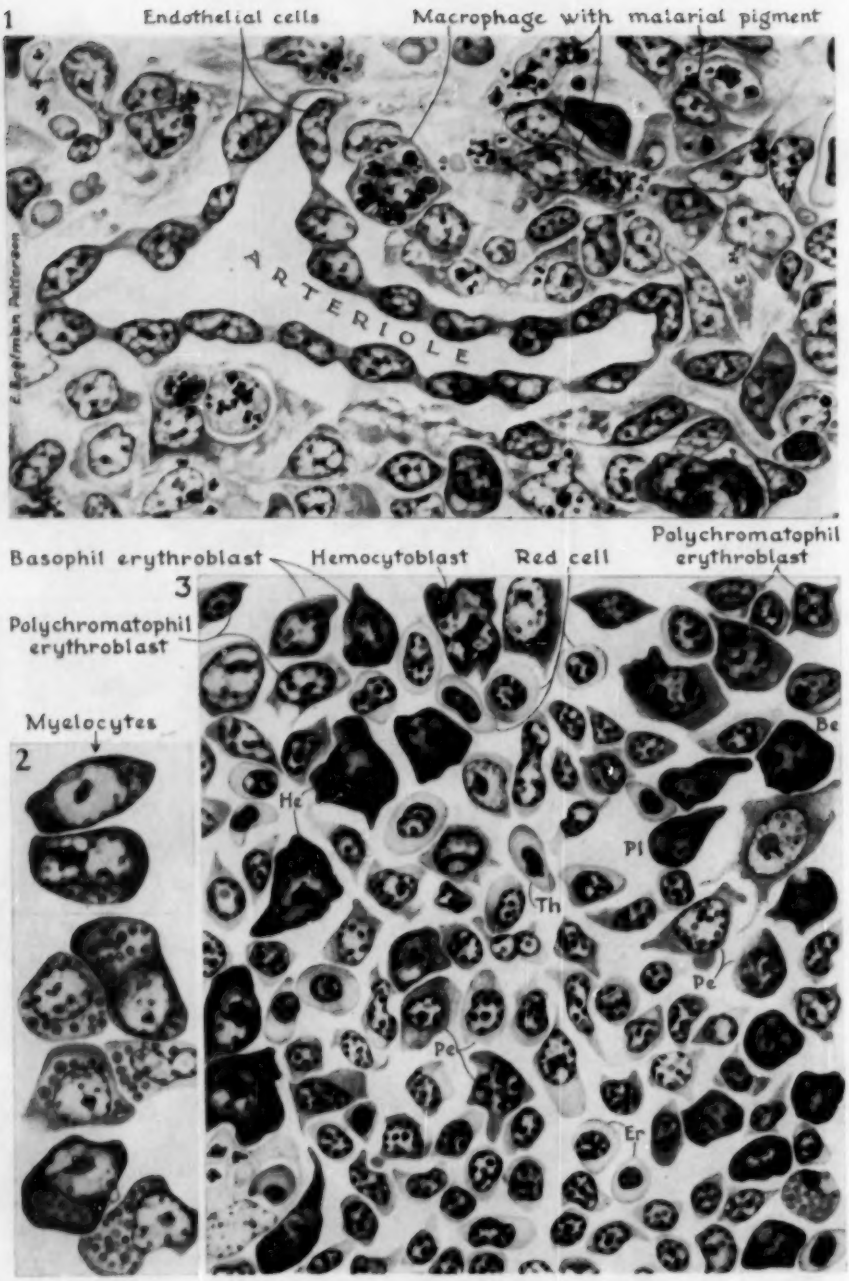


PLATE 6.—Drawings at the peak of the initial rise of *P. gallinaceum* infection. ×1900.

PL. 6, FIG. 1.—Macrophages containing malarial pigment in a Schweigger-Seidel sheath from a 5-day terminal infection (series 1). Note that the macrophages are less active than in the red pulp (pl. 5, fig. 2).

PL. 6, FIG. 2.—Cells transitional between large basophil lymphocytes (hemocytoblasts) and myelocytes in the periportal connective tissue of the liver from a 14-day infection (series 8).

PL. 6, FIG. 3.—A center of erythropoiesis in the red pulp of the spleen from a 14-day infection (series 8). Large hemocytoblasts (He) and basophil erythroblasts (Be) can be seen at the periphery of a collection of polychromatophilic erythroblasts (Pe) and young erythrocytes (Er). Th = thrombocyte.

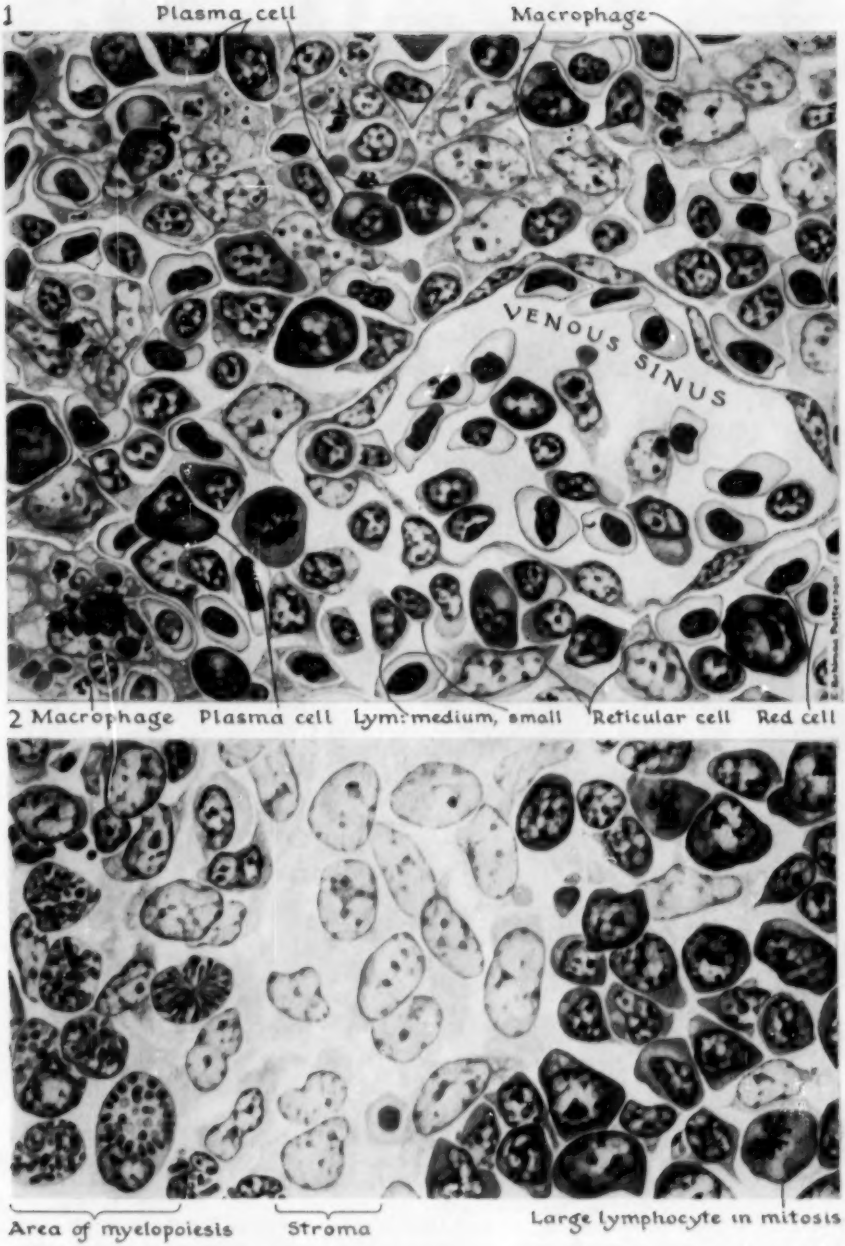


PLATE 7.—Drawings from the spleen and bone marrow after infection with *P. gallinaceum*. ×1900.

PL. 7, FIG. 1.—Spleen showing plasma cells and multinucleated macrophages in the Billroth cords from a 14-day infection just after the crisis (series 8). Note the dividing large lymphocyte.

PL. 7, FIG. 2.—Bone marrow showing a depletion of lymphoid cells (stem cells) and exposure of the reticular cells of the stroma from a 9-day terminal infection at the peak (series 7).

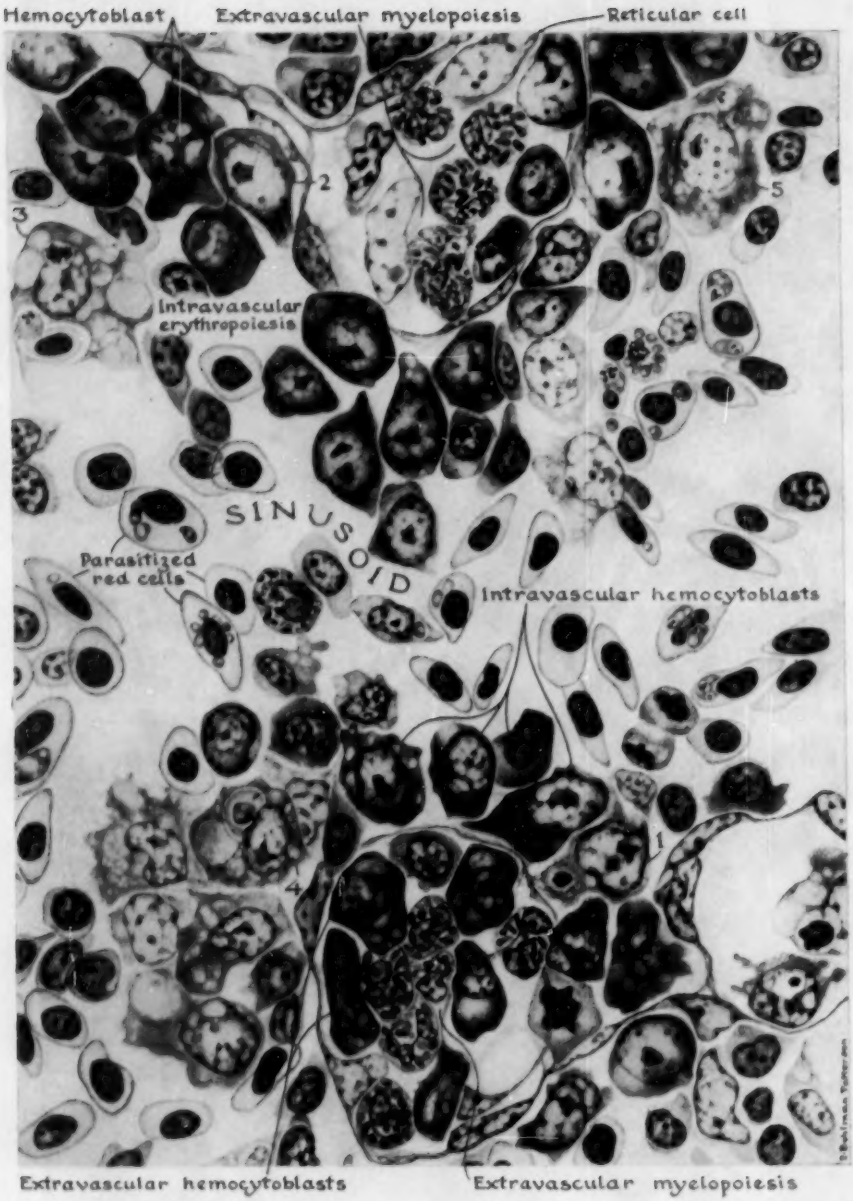


PLATE 8.—Drawing from the bone marrow at the peak of a 9-day terminal *P. gallinaceum* infection (series 7). Two small areas of extravascular myelopoiesis separated by a large area of intravascular (intrasinusoidal) erythropoiesis. Note that the hemocytoblasts which give rise to the erythropoietic series are aligned along the sinusoid wall and that a continuous series can be found between a typical hemocytoblast and a macrophage (cells numbered 1 to 5). Cell 4 contains a recognizable parasitized erythrocyte. Also note that many of the intra- and extravascular hemocytoblasts cannot be distinguished morphologically from the large basophil lymphocytes occurring elsewhere (cf. pl. 5, fig. 2; pl. 4, fig. 3 and pl. 7).

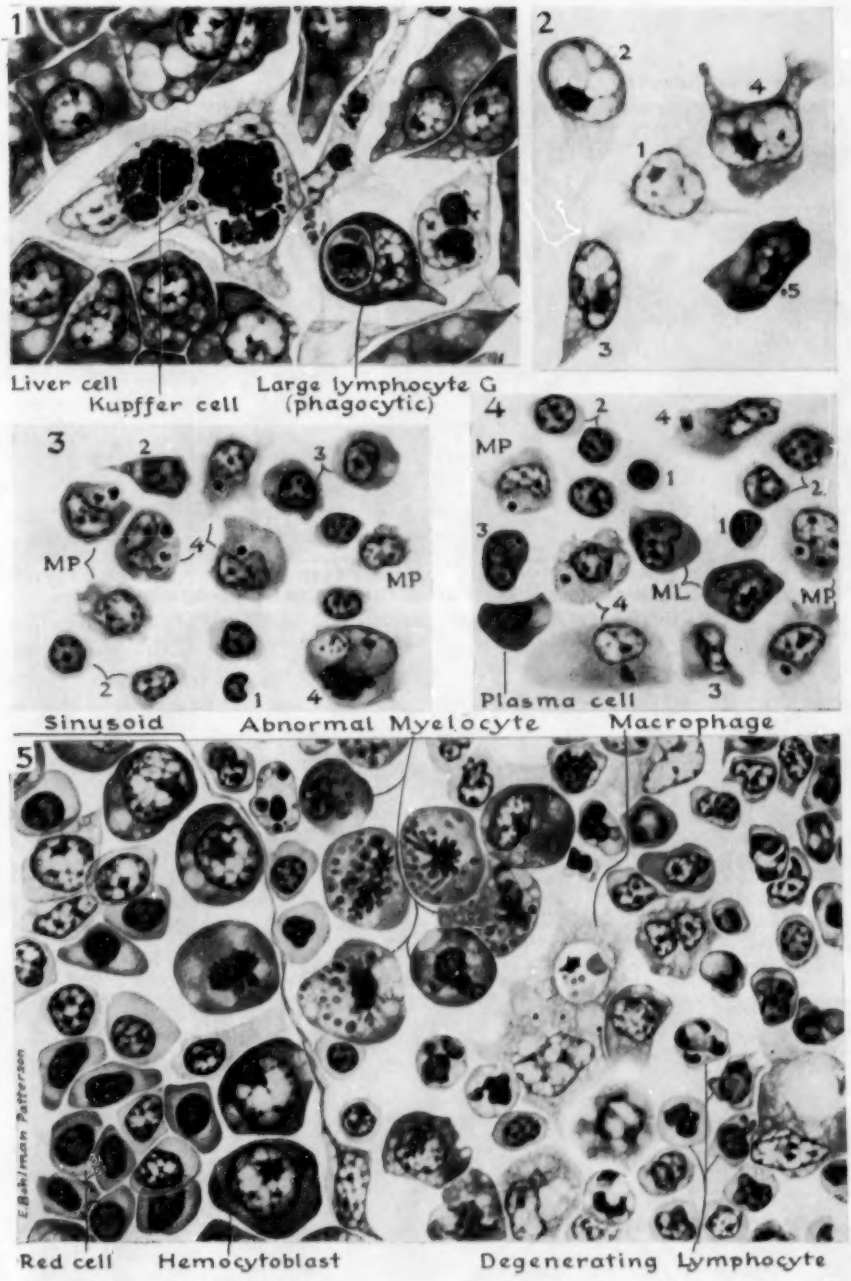


PLATE 9.—Drawings of tissues at the peak of *P. gallinaceum* infection. ×1900.

PL. 9, FIG. 1.—A phagocytic large lymphocyte containing a parasitized red cell and phagocytic Kupffer cells in a liver sinusoid of a 6-day terminal infection (series 1).

PL. 9, FIG. 2.—Transitional stages 1 to 5 from a reticular cell (=1) to a typical large lymphocyte (=5) in the spleen of an 8-day infection (series 7).

PL. 9, FIG. 3 and 4.—Early stages in the cytogenesis of macrophages from lymphocytes in the spleen from an 8-day infection (series 6). 1, a small lymphocyte; 2, medium lymphocyte; 3, monocytoid lymphocyte; 4, monocyte or monocytoid cell. ML, monocytoid lymphocyte derived from a large lymphocyte, and MP, medium polyblast.

PL. 9, FIG. 5.—Abnormal and dying cells in the bone marrow from a 4.5 day terminal infection (series 1). The intravascular hemocytoblasts, stages in the erythropoietic series and macrophages appear normal while some of the myelocytes and many of the lymphocytes show undoubted nuclear degeneration. One macrophage is phagocytic.

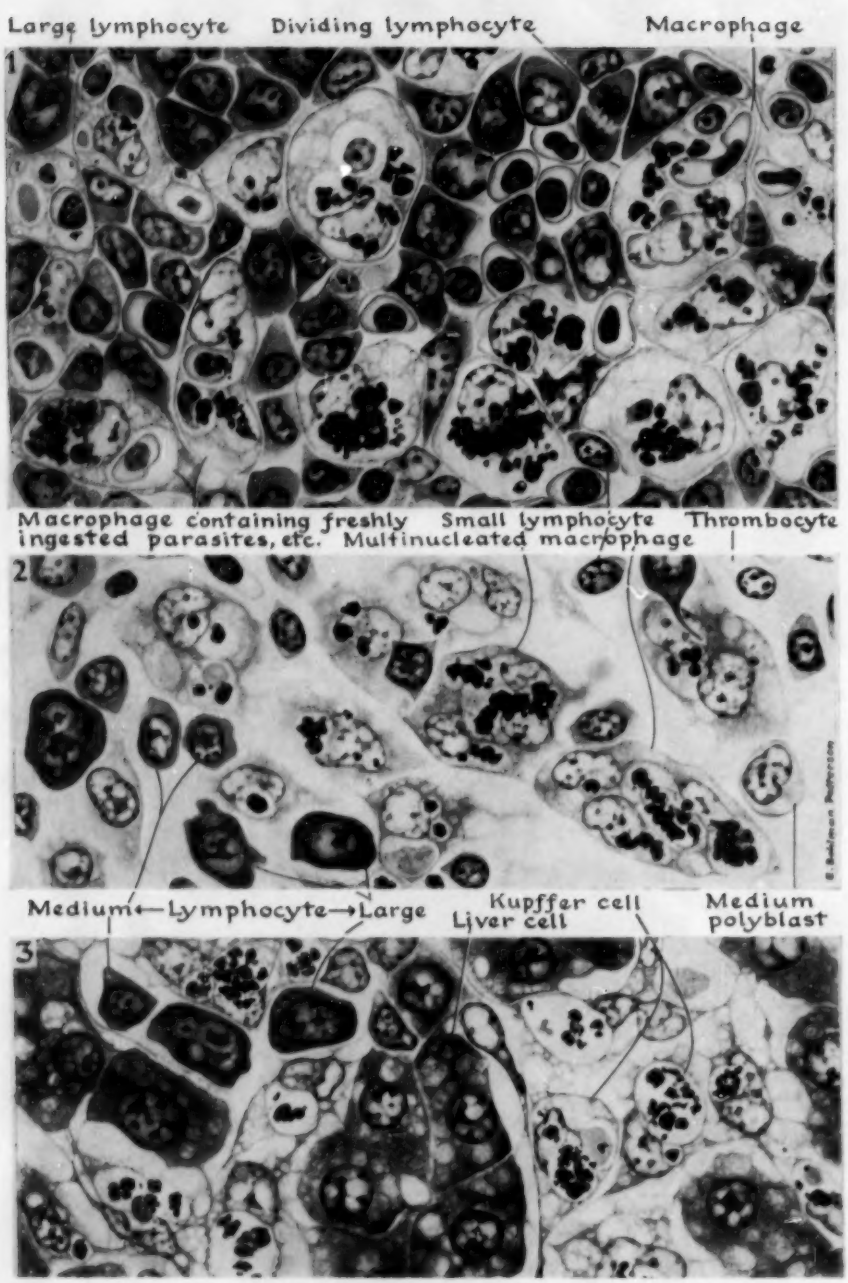


PLATE 10.—Drawings from the spleen and liver at the crisis of *P. lophurae* infection (series 17 and 18). ×1900.

PL. 10, FIG. 1.—Multinucleated macrophages and an unusually large number of large basophil lymphocytes in the Billroth cord of the spleen from a 6-day infection.

PL. 10, FIG. 2.—Cells, similar to those in figure 1, in the venous sinus from a 5-day infection.

PL. 10, FIG. 3.—Typical engorged Kupffer cells and vacuolated (fat) liver cells in the liver from a 6-day infection.

and the liver cells were abnormally vacuolated.

P. gallinaceum: initial infection.—In accord with our description of other malarias, the rise and fall of phagocytosis of parasites and debris by Kupffer cells closely followed the activity by splenic macrophages although the liver macrophages were individually less active than the splenic ones (cf. pl. 10, fig. 1 and 2 with 3). The speed with which the malarial pigment was redistributed, however, deserves special mention. It was particularly well seen when a crisis quickly reduced a high parasitemia to a low level. In such chickens by the end of the crisis, the majority of the littoral cells of the liver were filled with large masses of malarial debris and pigment (pl. 2, fig. 2). A day or so later, malarial pigment was found in fewer cells in larger masses. This tendency continued. Thus, the pigment was limited to about 5 masses per cross section of a liver lobule at 10 days whereas it was localized within the periportal connective tissue and to a less extent around the central vein or sinusoids leading from it at 50 to 60 days. In terminal infections, vacuolated liver cells and disorientation of the liver cords were found (pl. 2, fig. 3).

Lymphoid hyperplasia in the liver that could be ascribed to malaria was minimal. The type of cells found in the lymphoid accumulations, however, changed. Small lymphocytes markedly decreased and very large lymphocytes markedly increased. The latter cells during the acute rise when they were being mobilized from the reticulum in the spleen appeared in the sinusoidal circulation of the liver. Many divided, and an occasional one was phagocytic (pl. 9, fig. 1). They became more and more prominent in the periportal and local lymphoid accumulations during and after the crisis. During the developed infection, they were the predominant cell in many of the dense lymphoid accumulations where they were actively dividing. Later, many developed into plasma cells.

Extramedullary granulocytopoiesis occurred in the periportal tissue during the latter part of the acute rise and early part of the developed infection. In general, it was more extensive than in the spleen. The free stem cells mobilized partly from large lymphocytes and partly from stroma (reticular?) cells and probably adventitial cells of the dense lymphatic tissue in the periportal connective tissue. In support of the first origin, typical large intensely basophil lymphocytes were seen with a few intracytoplasmic myelocyte granules (pl. 6, fig. 2) as well as cells intermediate between them and typical myelocytes. In support of the second origin, mobilizing stroma cells were found with only a slight basophilia of the cytoplasm and with specific granulocyte granules. It seems probable from the sequence of events in other tissues that the large lymphocytes were recruited from the circulation, but their mobilization from outstretched stroma cells cannot be excluded.

For 6 months after the crisis, most chickens contained periportal or other lymphoid or myeloid accumulations in varying amounts from few to moderate (pl. 2, fig. 4). The large lymphocyte, however, markedly decreased in number, both relatively and absolutely.

Bone marrow

Uninfected chickens.—All bone marrow specimens were taken from the femur. Whenever possible, a long piece of marrow was removed and sectioned longitudinally so that comparisons could be made of areas near the epiphysis and at the edge and center of the long axis of the marrow. The chicken

bone marrow is particularly well suited for the differentiation of erythropoietic, granulopoietic and lymphocytopoietic activity. Thus, erythropoiesis is limited to the lumen of the sinusoids, granulopoiesis is localized to the extravascular parenchyma, and lymphocytopoiesis occurs in dense nodular and non-nodular lymphatic tissue usually associated with the arteries.

In our material, the normal bone marrow of adult chickens contained a large amount of fat except at the periphery and near the epiphyseal junction. Usually, there was not much erythropoiesis or granulopoiesis. Occasional small areas of dense lymphatic tissue often contained the smaller sizes of lymphocytes and at times a small nodule composed of varying numbers of small and medium lymphocytes (pl. 3, fig. 2). Somewhat more erythropoiesis and dense lymphatic tissue were found in young chickens.

P. gallinaceum: initial infection.—In agreement with reports on other malarias, phagocytic activity in the bone marrow was far less in every respect than in the spleen or the liver (cf. pl. 8 with pl. 5, fig. 2 and with pl. 9, fig. 1). At the time of the crisis, large macrophages, either singly or in small clumps in the sinusoids rarely contained more than one or two clumps of pigment. Some were probably developing from the large hemocytoblasts which line the walls of the sinusoids. Not only could all gradations in structure be found between these hemocytoblasts and macrophages (pl. 8) but cells containing parasitized erythrocytes or pigment clumps could be found which still retained most of the nuclear characteristics of the hemocytoblast and a decidedly basophil cytoplasm (pl. 8, cell 1). As time went on, the fine pigment granules coalesced into large clumps and were redistributed into fewer macrophages.

The malarial infection stimulated activity in all three classes of free marrow cells especially during the crisis (pl. 3, fig. 3). In most cases, the lymphocytes responded first, the red cell series second and the granulocyte series last. Increased amounts of dense non-nodular and nodular lymphatic tissue contained closely packed lymphocytes. Large areas of myelopoiesis appeared at the periphery of the lymphatic tissue throughout the cords. Occasionally, sinusoids were practically filled with stem cells, including large hemocytoblasts and basophil and polychromatophil erythroblasts. Many mitoses were found in all types of free stem cells, especially in large lymphocytes of the nodules, myelocytes and intrasinusoidal basophil erythroblasts which were giving rise to the erythrocyte series. It is of interest that the actively dividing precursor of the red cell was usually the basophil erythroblast rather than the large hemocytoblast. Associated with this intense bone marrow stimulation, cells indistinguishable from the very large lymphocyte of the spleen appeared (cf. pl. 8 with pl. 5, fig. 2). They apparently served as hemocytoblasts and gave rise to myeloblasts, erythroblasts and lymphocytes. As these activities increased, fat markedly decreased in amount, but only rarely disappeared. In moribund chickens, an intense lymphoid, myeloid and erythroid depletion of all free stem cells gave rise to an essentially aplastic marrow.

Both erythropoiesis and myelopoiesis remained at a high level for a month or more. Lymphocyte production compensated for lymphocyte depletion and at 1.5 months dense non-nodular and nodular accumulations contained actively proliferating large lymphocytes. From 2 to 6 months, regression varied depending upon the height of the continuing infection. When no phagocytosed pigment was found, the bone

marrow was essentially normal and possessed little, if any, extra lymphatic tissue. When pigment occurred, dense non-nodular lymphatic accumulations remained and contained a few lymphocytes in mitosis. At times, bare nodules were seen. This finding may indicate, in view of the fact that nodules were found in non-nodular lymphoid accumulations during earlier parts of the infection, that the lymphocytes originally surrounding the nodule died, migrated elsewhere or developed into other cells.

Bursa Fabricii

Uninfected chickens.—The bursa Fabricii comprises one of the largest single accumulations of dense lymphatic tissue. Each lobe was divided into well-defined cortical and medullary zones (pl. 3, fig. 4). The lighter appearance of the medullary zone was largely based upon the presence of fewer lymphocytes rather than a difference in the relative proportion of lymphocytes of a given size. All three sizes of lymphocytes occurred, and mitosis among all except the smallest ones appeared to be fairly frequent. This mitotic proliferation was accompanied by considerable lymphorrhexis with the ingestion of lymphocyte debris by macrophages.

P. gallinaceum: initial infection.—Little change was found in the bursa in many infections. Intense lymphoid depletion elsewhere, however, was reflected in the bursa by a disappearance of the boundary between the cortical and medullary zones, a marked disappearance of lymphocytes from the medullary zone, an abnormal amount of lymphorrhexis with phagocytosis and the focal accumulation of granulocytes in both the cortical and medullary zones. Sometimes, the medullary reticulum seemed almost denuded (pl. 3, fig. 5). Regeneration also occurred as evidenced by the enhanced mitotic proliferation of lymphocytes and the mobilization of large basophil lymphocytes from the medullary stroma.

Other tissues

Uninfected chickens.—Other tissues were normal in appearance. The local macrophages were neither swollen nor phagocytic. Some lymphocytes occurred in the intestinal wall. In an occasional chicken, there were a very few small circumscribed accumulations of lymphocytes of the smaller sizes in the pancreas, breast muscle or heart muscle.

P. gallinaceum: initial infection.—Changes in the lungs, thymus, kidney, pancreas, gut, muscle, brain and gonads were minor compared to those in the spleen, liver, bone marrow and bursa Fabricii. In general, as the parasitemia mounted, parasitized red cells were found in capillaries of various tissues. They even appeared to plug some capillaries during heavy infections. At times, they might mistakenly be considered to be phagocytosed by various cells such as endothelial cells in the brain which they closely abutted, but careful study revealed that phagocytosis was limited to intravascular free polyblasts. During intense parasitemias, many phagocytic macrophages occurred in the lungs. Some seemed to be fixed, others appeared to have wandered into the lungs from the capillaries and still others were intravascular. There was less phagocytic activity in other tissues. Some of the active macrophages were local: others were obviously circulating. Lymphoid depletion varied in amount even in intense and lethal infections. It was sometimes a prominent feature of the thymus. A few lymphoid accumulations and sporadically a nodule occurred in the lungs, pancreas, intestine and other tissues at the time of the crisis and thereafter (pl. 2, fig. 5). Such areas contained many large lymphocytes and eventually plasma cells in infections of long standing. Granulocytic infiltrations

and a few granulopoietic areas also occurred. For example, they were seen in the ovary and lungs of a 6-day terminal infection in series 1 and in the pancreas of a 9-day lethal infection in series 7. It was difficult to ascertain whether erythropoiesis was present. Pycnotic nuclei and other degenerative changes were found occasionally in various tissues.

Homologous superinfection with P. gallinaceum

The cellular changes during superinfection with *P. gallinaceum* were studied in 5 series (11 through 15) as outlined in table 3. The series consisted of 47 medium sized and 29 large chickens superinfected with blood parasites 1 to 2.5 months after the initial injection. The challenging dose of parasites was such that from 0.01 to 1 parasite per 10 red cells was found in the peripheral blood within 2 to 4 minutes after the injection. Such amounts resulted in nonlethal superinfections which reached a level of one parasite per 10,000 red cells or less in 1 to 4 days. The parasites used for the superinfecting doses were obtained by bleeding heavily infected chickens during the acute rise from the heart into citrated (0.4%) 0.85% NaCl and injecting immediately (without washing the cells free of serum) into the test chicken. This procedure preserved the viability of the parasites.

The parasitemias of all superinfections were graphed and the rapid disappearance of parasites has been described by us (1945 and 1950).

Spleen

Control immunized chickens.—The spleen of immunized chickens infected for 1 to 2.5 months showed the following general characteristics. Most of the malarial pigment was aggregated into large clumps. The red pulp contained many lymphocytes and large clumps of macrophages some of which were continuing their phagocytic activity because parasites continued to be present at barely patent levels. Erythropoiesis and myelopoiesis were prominent in some chickens. Dense non-nodular and nodular lymphatic accumulations usually in increased amounts were associated with depleted areas in which large lymphocytes were mobilizing from reticular cells. Many mitoses occurred in the nodules and to a less extent in the dense lymphatic tissue. The degree of phagocytosis, lymphoid depletion and hyperplasia, however, varied not only from series to series but from chicken to chicken in individual series. The effects

TABLE 3.—*Chickens superinfected with blood parasites of* P. gallinaceum *1 to 3 months after an initial injection with* P. gallinaceum. *One or two immune, i.e., not superinfected, chickens were killed in each series.**

Series	Number of chickens	Approximate weight in kg when superinfected	Nonlethal superinfection†			Used in text figure
			Parasite peak per 10 red blood cells	Day of peak	When killed	
11	19	1.0	0.1–0.2	—‡	5–19 days	4
12	10	1.0	0.4–1.0	1–2§	15 minutes– 7 days	4
13	20	0.5	0.1–0.5	—‡	5 minutes–32 days	4
14	9	0.6	2.0–4.0	1–2§	1 hour–5 days	4
15	8	0.5	1.0–4.0	1–2§	1 hour–3 days	4
16	12	0.7	Controls*			

* In addition to the immune control chickens, another control series was infected initially with large numbers of parasites and killed after 5 minutes to 2 days.

† Such a large number of parasites were given intravenously to superinfect these chickens that parasites appeared immediately in the blood.

‡ The parasitemia did not increase in series 11 and 13. Hence the peak was reached immediately after superinfection and parasites disappeared in 1 to 3 days.

§ Parasites lasted 4 to 7 days.

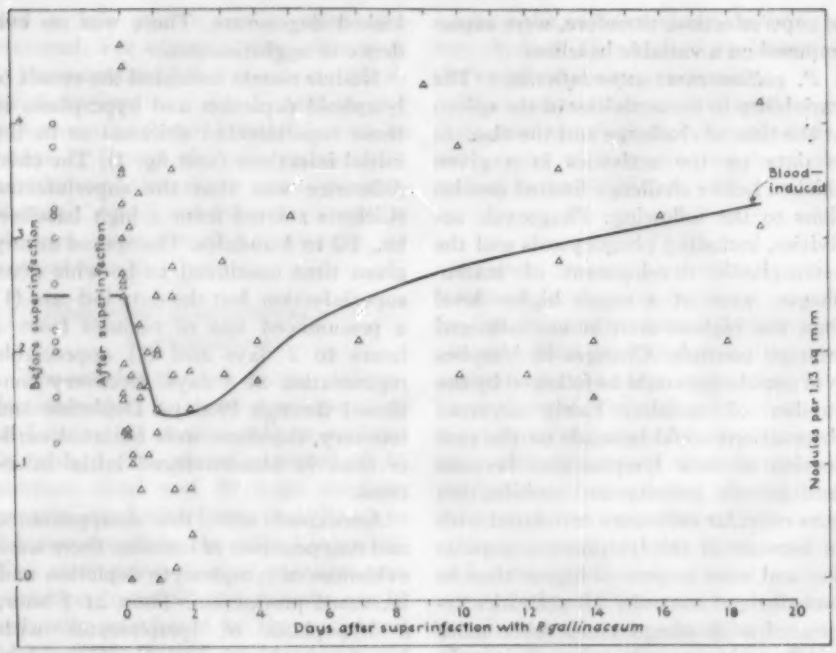
of superinfection, therefore, were superimposed on a variable baseline.

P. gallinaceum: superinfection.—The variability in the activities of the spleen at the time of challenge and the absence of data on the activities in a given chicken before challenge limited conclusions to the following: Phagocytic activities, including phagocytosis and the heteroplastic development of macrophages, were at a much higher level than the highest seen in unchallenged immune controls. Changes in lymphocyte population could be followed by the number of nodules. Fairly accurate observations could be made on the production of new lymphocytes because both mitotic activity and mobilization from reticular cells were correlated with an increase in the lymphocyte population and were in general higher than in unchallenged controls. All activities associated with phagocytosis were more rapidly evident and more efficient in getting rid of the parasites than in initially infected chickens.

The following timing was in general maintained. Undoubted changes were not apparent in the spleen during the first few minutes. Fresh malarial material and pigment began to be phagocytosed in 15 minutes, and the former began to be digested at 45 minutes. In 3 to 12 hours, the red pulp was full of swollen active macrophages. In heavy superinfections (series 14 and 15), masses of red cells (most of them parasitized) could be seen in individual macrophages. This enhanced activity was usually evident for a day or two and frequently included a widespread phagocytosis of granulocytes and lymphocytes. The exact duration of the heightened activity depended upon the size of the superinfecting dose. It regressed rapidly as the parasitemia settled back to the presuperinfection level. A few cyst-like macrophages looked degenerate. There was no evidence of agglutination.

Nodule counts indicated the extent of lymphoid depletion and hyperplasia in these superinfected chickens as in the initial infections (text fig. 4). The chief difference was that the superinfected chickens started from a high baseline, i.e., 1.2 to 4 nodules. The spread at any given time continued to be wide after superinfection, but the data indicate (1) a pronounced loss of nodules from 3 hours to 2 days and (2) appreciable regeneration at 3 days. Recovery continued through 19 days. Depletion and recovery, therefore, were initiated earlier than in blood-induced initial infections.

Associated with the disappearance and reappearance of nodules, there were evidences of lymphocyte depletion and increased production. Thus, at 1 hour, a hypoplasia of lymphocytes with lymphorrhexis was sometimes noted in the red pulp and areas of lymphocyte depletion were occasionally found in the perisheath and periarterial dense tissues. Lymphoid proliferation and mobilization probably began practically as soon as lymphoid depletion in heavy superinfections. What was probably an increase in mitosis occurred among the lymphocytes in the nodules as early as ½ hour in one chicken and at 3 hours in two others. By 9 hours, many dividing lymphocytes were present, especially in the nodules and dense non-nodular lymphatic areas. At 12 hours, large lymphocytes appeared to be mobilizing and granulocytes had increased in number. From 1 day through 36 hours, these activities were more clear-cut and, at 2 days, regenerative activities had gained the upper hand. At 3 days in one chicken, 48 mitoses were counted in one high power field in a nodule. These activities continued through 19 days after superinfection. The meager data



TEXT FIG. 4.—Nodules per 1.13 sq mm in the spleen for 19 days after superinfection with blood parasites of *P. gallinaceum*. Note the high baseline of nodules in immune chickens and, after superinfection, the rapid disappearance, reappearance and abnormally high level at 19 days.

after 19 days indicated that regression then took place.

Following a small challenging dose of parasites, phagocytosis was milder and the existing macrophages appeared more or less adequate to dispose of the parasites. There were, however, evidences of some lymphocyte depletion and a slight amount of lymphoid proliferation.

Myelopoiesis and erythropoiesis were initiated between 12 hours and 1 day, continued to be prominent for a week and then gradually decreased.

Liver, bone marrow and other tissues

P. gallinaceum: superinfection.—The more efficient removal of parasites in the spleen also occurred in the liver. Phagocytic Kupffer cells were active in proportion to the number of reinjected parasites. In addition, sporadic small lymphoid and/or myeloid accumulations were somewhat more prominent than in immune chickens.

Macrophages containing fresh malarial material were only rarely encountered in the bone marrow. Lymphoid, myeloid and erythroid hyperplasias were not noticeably above the level maintained in immunized unchallenged chickens.

No changes worthy of note occurred in the other tissues.

Initial infection with P. lophurae

The results of the study of *P. gallinaceum* infections were presented first because they were associated with the most striking reactions in the connective tissue. Fundamentally, however, the same changes were seen in lophurae in-

TABLE 4.—*Chickens initially infected or superinfected 1 to 2 months after an initial infection with blood parasites of* P. lophurae. *One to six control chickens were killed in each series.*

Series	Number of chickens	Approximate weight in kg when infected	Characteristics			Used in	
			Parasite peak per 10 rbc	Day of peak	When killed	Plate	Text figure
			A. Nonlethal initial infection of P. lophurae				
17	13	0.6	2–10	3–5	1– 5 days	10:2	5, 6
18	20	0.6	2–10	3–5	6–14 days	10:1, 3	5, 6
19	8	0.5	2–10	3–5	20–30 days		5, 6
20	35	0.5	2–10	3–5	1.25–4 months		5, 6
			*B. Nonlethal superinfection**				
		When superinfected					
21	33	0.6	1– 2	0–2	15 minutes–27 days		7
22	18	0.6	2– 3	2–3	15 minutes–17 days		7

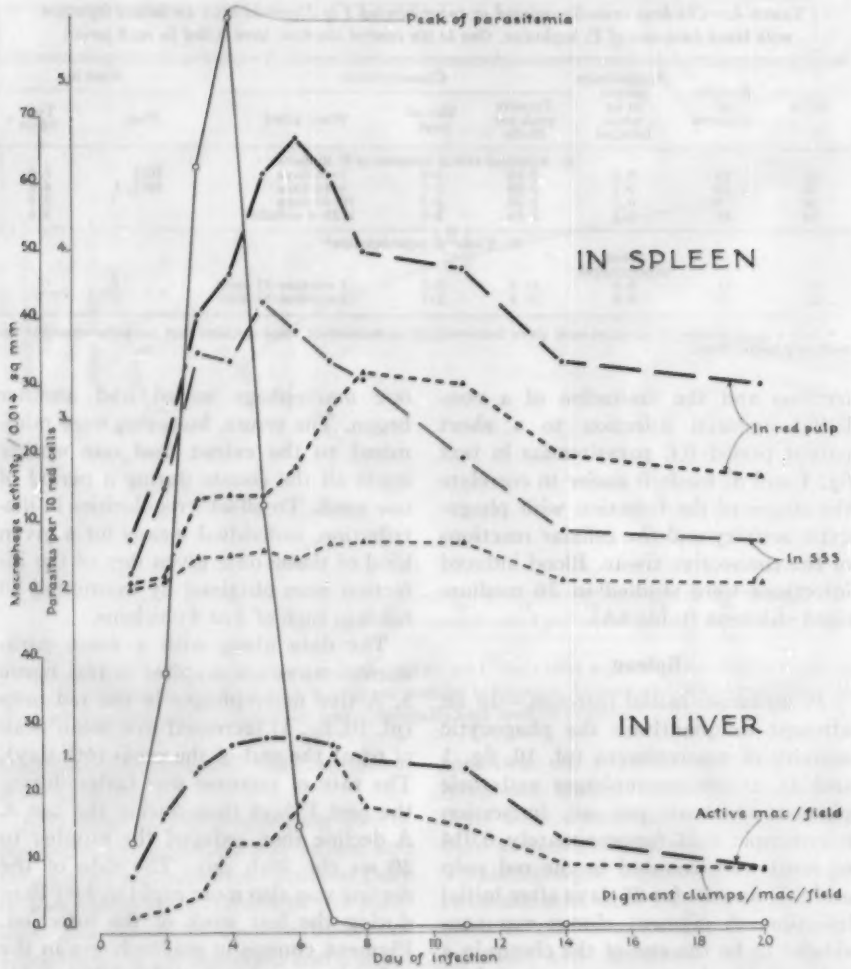
* Such a large number of parasites were given intravenously to superinfect these chickens that parasites appeared immediately in the blood.

fections and the limitation of a nonlethal malarial infection to a short patent period (cf. parasitemias in text fig. 1 and 5) made it easier to correlate the stages of the infection with phagocytic activity and the cellular reactions of the connective tissue. Blood-induced infections were studied in 76 medium sized chickens (table 4A).

Spleen

P. lophurae: initial infection.—In an attempt to quantitate the phagocytic activity of macrophages (pl. 10, fig. 1 and 2), active macrophages and their pigment content per oil immersion microscopic field (approximately 0.014 sq mm) were counted in the red pulp and S-S sheaths for 20 days after initial infection. A pigment clump was considered to be the size of the clump in a well-developed mature segmenter. In a given sample, small granules were added from several macrophages and large masses in a single macrophage were divided to attain the approximate number of pigment clumps of the designated size. Estimate of the large masses was particulary troublesome and may have been far too small, especially for macrophages after the crisis. In fact, a similar study of *P. gallinaceum* infections had to be abandoned for this reason. Sometimes it was difficult to determine where one macrophage ended and another began. The errors, however, were minimized to the extent that one worker made all the counts during a period of one week. To offset irregularities in distribution, individual means for a given kind of tissue on a given day of the infection were obtained by examining 20 fields in each of 3 or 4 chickens.

The data along with a mean parasitemia curve are graphed in text figure 5. Active macrophages in the red pulp (pl. 10, fig. 1) increased to a mean peak of 68 at the end of the crisis (6th day). The rate of increase was faster during the first 3 days than during the last 3. A decline then reduced the number to 30 on the 20th day. The rate of the decline was also more rapid at first than during the last week of the infection. Pigment clumps in macrophages in the red pulp were first found in appreciable numbers on the 3rd day of the acute rise. The rise in phagocytosed pigment from the 5th to the 8th day when active macrophages were decreasing in number and when the parasitemia was of a low grade was probably due to the death of engorged macrophages with a redistribution of the malarial debris to other phagocytes. Similarly, the decline to 17 clumps per macrophage per field by the 20th day may have represented in part the digestion of some pigment and in



TEXT FIG. 5.—Macrophages containing malarial pigment per 0.014 sq mm in the red pulp and S-S sheaths of the spleen and in the liver for 20 days after initial infection with blood parasites of *P. lophurae*. In addition, pigment clumps per macrophage per 0.014 sq mm in the same three sites. Note the differences in the rise and fall of these two measures of phagocytic activity with respect to the parasitemia curve.

part a further redistribution. Some pigment clumps were in multinucleated macrophages. Others appeared at times to be extravascular. The active macrophages and the mean pigment clumps each macrophage contained in the S-S sheaths were less than in the red pulp (text fig. 5), but followed the same general course except that the peak in active macrophages was reached a day earlier (43 on the 5th day) and the peak of the pigment content per active macrophage only reached 6 per field. Macrophages were inactive in the nod-

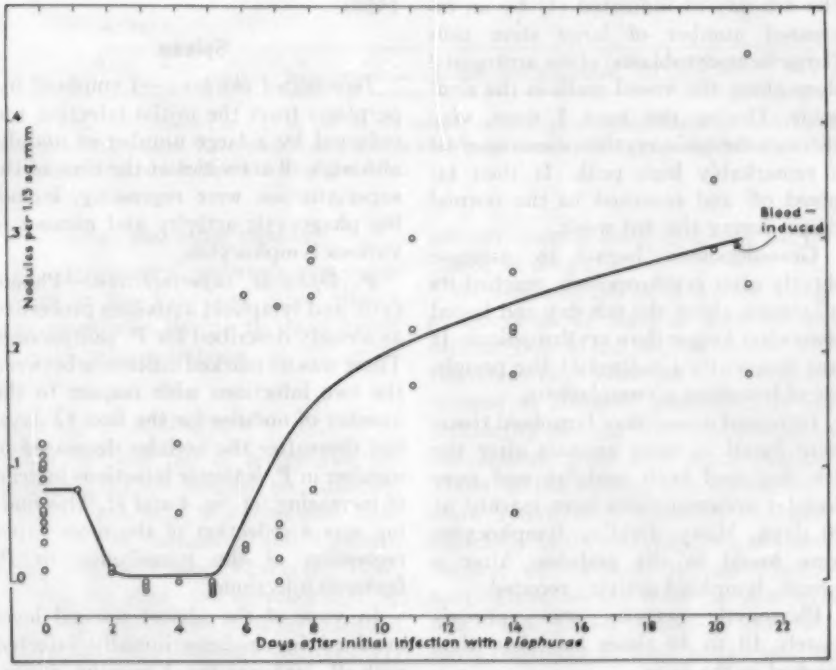
ules and ordinarily contained merely small pigment clumps or a few pigment granules.

The number of nodules, graphed in text figure 6, illustrates the milder and more transient character of this infection as compared to *P. gallinaceum* infection. Briefly, the nodules reached a low ebb on the 2nd through the 5th days, reappeared on the 7th and 8th days and attained an approximate 4-fold increase over normal at 3 weeks. Thereafter, they gradually declined to a normal value at 3 months.

As compared to initial blood-induced infections with *P. gallinaceum*, nodules disappeared practically as fast but reappeared a week sooner, reached a peak almost a month sooner, began to recede 2 months sooner and reached the normal value several or more months sooner.

Liver

P. lophurae: initial infection.—Changes in the liver were indistinguishable from those described for *P. gallinaceum*. Various degrees of irregular fatty necrosis occurred between the 4th and 6th day of the infection (pl. 10, fig. 3). Lymphatic nodules and nonnodular accumulations were in general more numerous at the end of the crisis on the 7th day than in normal chickens, but were not consistently present during the postcrisis period. Focal areas of



TEXT FIG. 6.—Nodules per 1.13 sq mm in the spleen for 21 days after initial infection with blood parasites of *P. lophurae*. Note their initial disappearance, more rapid reappearance and higher level at 21 days than in *P. gallinaceum* infections. Thereafter, nodules decreased in number and were practically within the normal range at 3 months.

granulopoiesis usually could be seen during the 2nd and 3rd weeks.

The phagocytic activity of Kupffer cells and the quantity of pigment they contained were correlated with the course of the infection (text fig. 5). In general, the rise and fall of these two activities more nearly approximated the sequence encountered in the red pulp than in the S-S sheaths except that peaks were smaller and were reached a day sooner.

Bone marrow

P. lophurae: initial infection.—Reactions in the bone marrow also closely followed the changes described for gallinaceum infections. Undoubted stimulation of erythropoiesis was found by the 4th day as indicated (1) by an increased number of large stem cells (large hemocytoblasts) often arranged 3 deep along the vessel walls in the sinusoids. During the next 5 days, viz., through the crisis, erythropoiesis reached a remarkably high peak. It then tapered off and returned to the normal range during the 3rd week.

Granulopoiesis began to increase shortly after erythropoiesis, reached its maximum about the 6th day and lasted somewhat longer than erythropoiesis. It was frequently localized at the periphery of lymphoid accumulations.

Increased amounts of lymphoid tissue were found in most animals after the 4th day and both nodular and nonnodular accumulations were marked at 20 days. Many dividing lymphocytes were found in the nodules. After a month, lymphoid activity receded.

Phagocytic activity was approximately 10 to 40 times less than that reached in the liver.

Other tissues

P. lophurae: initial infection.—Reactions in other tissues were minimal. They were consistently milder than those described for *P. gallinaceum*.

Homologus superinfection with P. lophurae

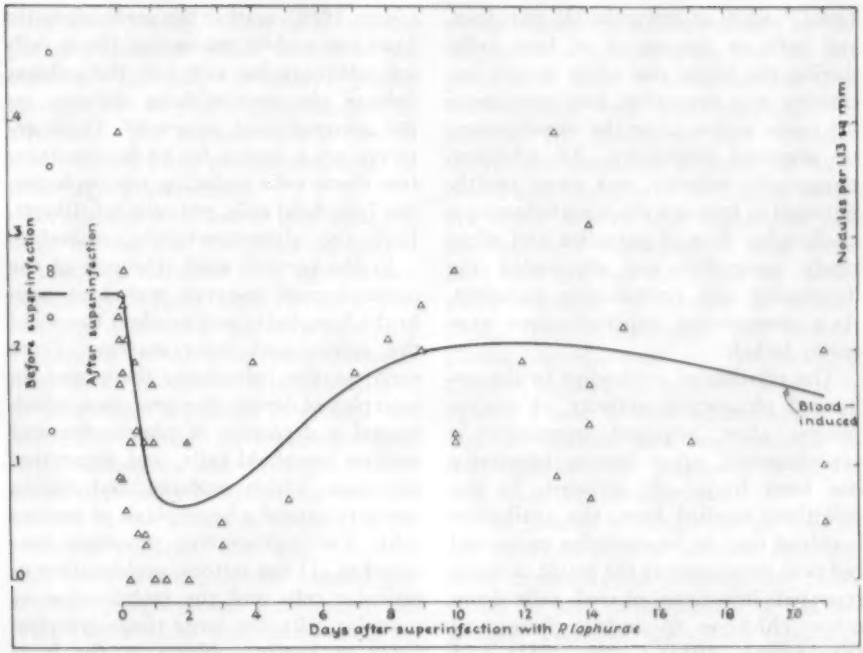
Various activities following a large challenging intravenous dose of *P. lophurae* were examined in a total of 51 medium sized chickens (table 4B). The superinfection was nonlethal and was studied at intervals from 15 minutes to 27 days. The chickens had been infected initially from 1 to 2 months earlier. Parasites either did not increase at all (peak on the 0 day) or else reached a peak on the 1st or 2nd day and disappeared by the 3rd to 5th day. These aspects of the superinfection have already been described by us (1945 and 1950).

Spleen

Immunized chickens.—Lymphoid hyperplasia from the initial infection was reflected by a large number of nodules although all activities at the time of the superinfection were regressing, including phagocytic activity and mitoses in various lymphocytes.

P. lophurae superinfection.—Phagocytic and lymphoid activities proceeded as already described for *P. gallinaceum*. There was no marked difference between the two infections with respect to the number of nodules for the first 12 days, but thereafter the nodules decreased in number in *P. lophurae* infections instead of increasing (cf. fig. 4 and 7). This finding was a reflection of the more rapid regression of the parasitemia in *P. lophurae* infections.

In view of the almost normal level attained in chickens initially infected with *P. lophurae* for 3 months, superinfection at 3 months or shortly thereafter would probably have elicited more uniform activities than we found in superinfections at 1 to 2 months.



TEXT FIG. 7.—Nodules per 1.13 sq mm in the spleen for 21 days after superinfection with blood parasites of *P. lophurae*. Note their disappearance and reappearance, and quicker regression after 15 days than after superinfection with *P. gallinaceum*.

Liver and other tissues

P. lophurae: superinfection.—Activities in the liver and other tissues were essentially similar to those found in mild superinfections of *P. gallinaceum*.

DISCUSSION

The rise and fall of phagocytic activity in earlier studies on canaries, monkeys and mice (Cannon and Taliaferro, 1931; Taliaferro and Cannon, 1936; Taliaferro and Mulligan, 1937; and Singer, 1954) are corroborated by the present work and need no extended discussion. The interplay between (1) the loss and production of macrophages and lymphoid cells and (2) the importance of phagocytic and mesenchymal reserves has been carried further by the present investigation and will be discussed in detail.

As shown in the studies cited above, phagocytosis of malarial material and cellular debris can be correlated with the intensity of infection and the development of acquired immunity. It was practically limited to macrophages of the spleen, liver and bone marrow. Those of the spleen were individually more active than those of the liver, especially in the red pulp, but the liver, because of its size, is more important. Those in the bone marrow were much less active. Although they differed relatively in their phagocytic activity in the three sites, they showed the same general trends (cf. the activity in the spleen and liver in text fig. 5). They phagocytosed pigment and "fresh ma-

terial," such as recognizable parasites, red cells or fragments of host cells, during the acute rise while innate immunity was operative, but were more intensely active after the development of acquired immunity. In addition, phagocytic activity was more swiftly initiated in immune chickens following a challenging dose of parasites and effectively controlled and eliminated the developing and reproducing parasites. As a consequence, superinfections were rarely lethal.

The relation of antibodies to the enhanced phagocytic activity of macrophages after acquired immunity is superimposed upon innate immunity has been frequently stressed. In the infections studied here, the antibodies involved may be iso-opsonins to normal red cells developed as the result of using repeated injections of red cells from other chickens to induce immunity (Zuckerman, 1945; Coffin, 1951a and b; and Swink, 1954) as well as specific agglutinins and opsonins (Trager et al, 1950; Stauber et al, 1951; Ben Harel, 1951; and Coffin, 1951). In this connection, reference should be made to the work of Becker et al (see review in 1952). These authors imply an antibody basis for the phagocytosis involved in the early removal of parasites. When duck erythrocytes infected with *P. lophurae* were injected into immunized chickens, they found that a previous injection of duck serum or plasma partially inhibited phagocytosis. This "sparing phenomenon," they believe, is due to an antigen-like substance which inhibits antibody-induced phagocytosis.

Emphasis on the phagocytic aspects (both cellular and humoral) of malarial immunity has often tended to obscure the importance of the mesenchymal reserves in both the antiparasitic and reparative aspects of immunity. As pointed out in a recent review (Taliaferro, 1949), neither the predominantly fixed nor mobile connective tissue cells are adequate for any but the mildest defense reactions without drawing on the mesenchymal reserves.* These reserves are a source for various connective tissue cells including macrophages, free lymphoid cells, red cells (cf. Bloom, 1940) and, almost certainly, antibodies.

In the present work, the role of the mesenchymal reserves was best seen in the lymphatic and myeloid tissues of the spleen and bone marrow. From early in the infections, there was an interplay of destructive processes, which caused a depletion of phagocytes and various lymphoid cells, and reparative processes, which replaced and during recovery caused a hyperplasia of various cells. The regenerative processes consisted of (1) the mitotic proliferation of reticular cells and the mobilization of reticular cells into large tissue lymphocytes or hemocytoblasts in the lymphatic and myeloid tissues and (2) the mitotic proliferation of lymphoid cells. These processes will be considered in detail.

Overengorged macrophages often became cyst-like and abnormal. At such times, many died and disintegrated. Then they and their contents were taken up by other macrophages. This process was particularly apparent in lethal infections when little acquired immunity developed and in nonlethal

* The cells included in the mesenchymal reserves are listed by Taliaferro (1949). In the present infections, the predominantly fixed reserves include the reticular cells of the spleen and bone marrow and the littoral cells of the liver. They are part of Aschoff's reticulo-endothelial system in the restricted sense. The free or mobile mesenchymal reserves include the various lymphoid cells of the blood and reticular tissues, such as lymphocytes, hemocytoblasts and other early "blasts." All of these fixed and free cells are included in the lymphoid-macrophage system of Taliaferro and Mulligan (1937).

infections during crises and partial parasite declines. Thus, much of the ingested material probably passed through two or more macrophages. In any case, the marked destruction of macrophages was a pronounced drain on the phagocytic reserves.

In addition to a loss of macrophages, large numbers of lymphocytes, granulocytes and red cells were injured and eventually phagocytosed. Dividing lymphocytes of medium size were especially vulnerable. On the other hand, the series from hemocytoblast to erythroblast and to myeloblast remained in general unharmed (pl. 9, fig. 5). Somewhat similarly, Taliaferro and Mulligan (1937) found that many lymphocytes were destroyed in the spleen in terminal acute knowlesi malaria, but that reticular cells remained apparently normal and actively phagocytosed lymphocyte debris. The intense lymphocytocidal action and the lesser damage to the early cells of the granulocyte series by these malarias are similar to the more marked damage by ionizing radiations (cf. Bloom and collaborators, 1948; and W. H. and L. G. Taliaferro, 1951). In view of the role of lymphocytes (and other lymphoid cells) in the heteroplastic development of macrophages, this lymphocytocidal activity is antagonistic to the development of effective phagocytic immunity and depletes the mesenchymal reserves. It can be classed as an "aggressive factor" in the parasitic invasion of the host.

The "demand" for macrophages was met at first by the reticular cells of the spleen and bone marrow and the littoral cells of the liver which can become immediately phagocytic. Then, new macrophages arose to a small extent by the mitotic division of reticular cells but mainly by the heteroplastic development from lymphocytes and monocytes. The sequence of intense lymphocytopoiesis in the spleen followed by the migration of lymphocytes into the red pulp and their transformation into macrophages was first suggested in malaria by Taliaferro and Cannon (1936) and described in detail by Taliaferro and Mulligan (1937). Most workers today believe that monocytes can develop into macrophages, and some hold that the two cell types are essentially identical (cf. review in Bloom, 1938). The hypertrophy and heteroplastic development of lymphocytes through monocytes, as shown in plate 9, figures 3 and 4, into macrophages as well as directly into macrophages as first described by Maximow (1902) is still, however, controversial.

The present investigation as well as our previous studies indicate that the fixed mesenchymal reserves are functional in the development of macrophages. Those of the spleen (reticular cells) are greatly increased in total number as the spleen hypertrophies. More important, however, are the mobile mesenchymal reserves (lymphoid cells) of the spleen and bone marrow. The free cells replace the cells destroyed by the malarial infections such as macrophages (for the most part), lymphocytes (except for those arising by transformation from reticular cells), granulocytes, and red cells; they supply the greater part of the increased number of lymphoid cells which give the immune animal an added cushion during superinfection; and they, possibly in conjunction with the fixed cells, probably form antibodies.

We have no evidence as to how the cellular reticulum is regenerated after it loses reticular cells. The latter transform into free macrophages and large lymphocytes—sometimes in large numbers. The comparatively rare occurrence of mitoses in reticular cells suggests that these cells may also be largely

TABLE 5.—*Mean number of nodules/1.13 sq mm illustrating lymphoid depletion (B) and lymphoid hyperplasia (C and D) as contrasted to normal or immune chickens (A).**

	Initial infection		Superinfection	
	P. gallinaceum: blood-induced			
A	Normal	0.80±0.09	Latent–3 hours	2.70±0.57
B	1–13 days	0.19±0.04*	4 hours–2 days	1.65±0.35
C	15–30 days	1.90±0.24*	3–30 days	2.61±0.58
D	1–2.5 months	2.84±0.13*		
	P. gallinaceum: sporozoite-induced			
A‡	Normal	0.80±0.09		
B_1‡	1–4 days	0.77±0.15		
B_2	5–17 days	0.28±0.04*		
C	20–30 days	1.78±0.20*		
D	1–2.5 months	1.95±0.12*		
	P. lophurae: blood induced			
A	Normal	0.80±0.09	Latent–3 hours	2.38±0.27
B	1–5 days	0.22±0.09*	4 hours–3 days	0.89±0.18*
C	8–30 days	2.35±0.54†	4–30 days	1.77±0.18†
D	1–2.5 months	1.87±0.16*		

* The significance of the differences between A and corresponding B, C and D groups are indicated by the following probabilities.
* $P \leq 0.001$, † $P = 0.006$.
‡ See delay in disappearance of nodules in text figure 3.

replaced by cells which originally developed from agranulocytes.

In severe infections, such as the malaries studied in the present work, it is not surprising that, although lymphoid cells start to proliferate early, the lymphoid mesenchymal reserves become depleted at first, later regenerate as recovery ensues and eventually over-regenerate during continued low grade infection. Such an interplay between lymphocytopoiesis and lymphocyte depletion was first clearly described by Conway (1938 and 1939) in her work on *Listeria monocytogenes*. It is well-illustrated by the rise and fall of nodules in the spleen. For example, the number of nodules reflected (1) the initial depletion and subsequent regeneration to above normal levels of lymphatic tissue in initial infections of *P. gallinaceum* and *P. lophurae* (text fig. 2, 3 and 6) and in superinfections* of *P. lophurae* (text fig. 7), (2) the differences between sporozoite- and blood-induced infections (text fig. 3), (3) the intensity of *P. gallinaceum* as compared to *P. lophurae* initial infections (cf. text fig. 2 and 6), and (4) the milder nature of superinfections as contrasted to initial infections due to the existence of acquired immunity (cf. text fig. 2 with 3 and 6 with 7).

Salient data on the mean number of nodules and the statistical significance of differences in various categories from corresponding controls are shown in table 5. Moreover, the following trends are noteworthy. (1) There were only 1.95±0.12 nodules in the sporozoite-induced infections as compared to 2.84±0.13 nodules in the blood-induced infections at 1 to 2.5 months. These findings may be associated with the greater drain on the lymphoid reserves by the sporozoite- than by the blood-induced infection. The greater death rate in the one than in the other during initial infection supports this point of view. (2) The number of nodules after superinfection did not surpass the pre-superinfection level (2.61 vs. 2.7 nodules for *P. gallinaceum* and 1.77 vs. 2.38 nodules for *P. lophurae*) as in initial in-

* The lack of a statistical difference for depletion after superinfection with *P. gallinaceum* was unquestionably associated with variability in the immune baseline (see table 5).

fections (1.9 vs. 0.8 in *P. gallinaecum* and 2.35 vs. 0.8 in *P. lophurae*). This difference was probably in part due to the more rapid and more effective suppression of the superinfection. The possibility exists, however, that a hyperplasia above the level in immune birds may have developed in superinfections with *P. gallinaceum* had observations been continued longer.

Changes in the number of nodules gave the most satisfactory over-all results, but other differences were encountered. Thus, nodules tended to be very small as they reappeared and were often large (>0.01 sq mm) for a month or so. Similarly, mitoses within the nodules were more numerous during recovery and overstimulation than in normal chickens.

It is interesting that severe lymphocyte depletion in the spleen resulted in the virtual disappearance of lymphocytes from the non-nodular lymphatic tissue which had previously developed around the Schweigger-Seidel sheaths as a result of infection. As a consequence, the cellular reticulum in these areas became clearly visible. Such areas are often ascribed to RES hyperplasia in the literature. Actually, they result from a loss of lymphocytes without any necessary increase in reticular cells. Taliaferro and Pizzi (1955) found comparable areas of lymphocyte depletion in the transitional zone at the edges of the lymphatic nodules in mice infected with *Trypanosoma cruzi*.

Associated with the stimulation of the lymphoid system, small lymphocytes relatively decreased and the larger sizes increased (cf. pl. 5, fig. 2 with fig. 1). Also, there was a striking difference in the lymphocytes involved in initial and later regenerative lymphoid hyperplasia. Medium and large tissue lymphocytes proliferated mitotically at first. Later, after lymphocyte depletion, masses of unengorged (probably mainly fixed) reticular cells transformed into very large tissue lymphocytes. This is the cell, which, as previously pointed out, has been given many names. In current literature, it is most frequently considered to develop into plasma cells. Our own work indicates that it is a primitive cell of marked mesenchymal potencies which appears in response to severe infections (and other drains on the mesenchymal reserves). For example, Taliaferro and Mulligan (1937) furnished evidence that in malaria in rhesus monkeys it not only divided to form lymphocytes but developed heteroplastically in the spleen to form macrophages (see also Allen and Spitz, 1945) and in the sinusoids of the adrenal to form erythroblasts. In the present work, it divided even more frequently to form lymphocytes, developed into plasma cells and seemed to replace the usual hemocytoblast in the medullary and extramedullary development of all types of bone marrow cells. In the bone marrow, it appeared to develop heteroplastically into macrophages. It is also of interest that this very large lymphocyte with no observable morphological change was occasionally phagocytic (pl. 9, fig. 1).

Although conclusive evidence indicates that antibodies can be formed in the spleen and lymph nodes (review by Taliaferro, 1949), the cell or cells involved are still undetermined. Recently, considerable evidence has accumulated indicating that the plasma cell series (the large tissue lymphocyte noted above, the typical Marschalkó plasma cell and cells of intermediate structure) either form or store antibodies (see especially Coons et al, 1955; Fagraeus, 1948 a and b; Kolouch et al, 1947; and review by Taliaferro, 1949). This conception intensifies interest in the widespread mobilization and proliferation of

the very large tissue lymphocytes in chicken malaria. It should be noted, however, that even if future work indicates that this cell does not form antibody or that another cell is more important, it seems highly probable that some cell of the lymphoid-macrophage system is involved.

The immunological aspects of the present work have been limited to defense against the blood stages of *P. gallinaceum* and *P. lophurae*. Some mechanism is undoubtedly effective against the exoerythrocytic stages because they are generally greatly reduced in number or disappear after acquired immunity reduces the patent blood infection. Although we saw no histological evidence of the destruction of exoerythrocytic stages, we believe immunity must be acquired against them.

CONCLUSIONS

Connective tissue changes were studied in various tissues taken from chickens at closely spaced intervals throughout the course of infection and superinfection with *Plasmodium gallinaceum* and with *Plasmodium lophurae*. The changes took place chiefly in the spleen and to a less extent in the liver, bone marrow and bursa Fabricii. Little or no reaction occurred in other tissues.

All activities were roughly correlated with the rise and fall of the parasitemia in initial infections and superinfections and chiefly involved (1) the phagocytosis of parasitized red cells, parasites and parasite debris including malarial pigment by macrophages, chiefly of the spleen, liver and bone marrow, (2) the destruction of lymphocytes and other lymphoid cells, macrophages and granulocytes; (3) the mitotic proliferation of lymphoid cells in lymphatic and myeloid tissues, (4) the marked increase of abnormally large lymphocytes by mobilization from reticular cells and mitotic proliferation, (5) the development of plasma cells, and (6) the production of macrophages homoplastically from reticular cells and heteroplastically from lymphocytes (and hemocytoblasts) and monocytes. All of these activities were more stereotyped in mild than in intense persisting initial infections and were initiated more rapidly in superinfections.

Special attention was paid to the role of the mesenchymal reserves (both fixed and free cells) in the immune reactions. Among the free mesenchymal reserves there was an interplay between the loss and production of lymphoid cells, especially in the spleen and bone marrow. Thus, during the first part of initial infections, a progressive depletion of lymphocytes in the spleen reached its maximum in terminal lethal infections and was associated with the degeneration of many lymphocytes and the development of others into macrophages. Production of lymphocytes, which began early, compensated for this loss and produced a lymphoid hyperplasia during the late acute rise in mild infections and during the developed infection in persisting infections. The hyperplasia lasted a month or more. Regression depended upon whether the infection subsided. The lymphoid hyperplasia involved a mitotic proliferation of lymphocytes, especially medium and large ones and, to a less extent, the mobilization of the very large ones from reticular cells. The very large lymphocytes frequently developed into plasma cells and appeared to be a primitive free cell which often migrated to various tissues and played a major role in the development of various connective tissue cells associated with malarial immunity and repair. After superinfection, lymphoid depletion and lymphoid hyperplasia were initiated sooner and subsided more rapidly. Less pronounced changes occurred in erythro-

poietic and myelopoietic centers.

Lymphoid activities were paralleled in a remarkable manner by the number of nodules in the spleen. Thus, the disappearance and reappearance of nodules reflected differences between sporozoite- and blood-induced initial infections of *P. gallinaceum* and between blood-induced initial infections and superinfections of *P. gallinaceum* and *P. lophurae*.

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REACTIONS OF THE CONNECTIVE TISSUE IN CHICKENS TO *PLASMODIUM GALLINACEUM* AND *PLASMODIUM LOPHURAE*

II. GLUCOSE METABOLISM DURING INITIAL INFECTIONS

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Part I of this series (W. H. and L. G. Taliaferro, 1955) describes the histological reactions occurring in the connective tissue of chickens during initial infection and superinfection with *Plasmodium gallinaceum* and *Plasmodium lophurae*. The aim of the present study was to ascertain if the extensive changes in the connective tissue of infected chickens, especially during the immunological reactions associated with the parasitological crisis, are accompanied by changes in the metabolism of the host tissues. Spleens from chickens infected with *P. gallinaceum* were used for most of the work because the most pronounced tissue changes were observed in this organ. The rates of aerobic and anaerobic glucose metabolism in organ slices were measured as convenient indices of the general level of metabolic activity in the host tissues. A preliminary report of this work was published by Taliaferro and Moulder (1951).

MATERIALS AND METHODS

Parasitological methods.—All procedures relating to the maintenance of the two malarial species in chickens were the same as in Part I. Whenever tissues were used for biochemical measurements, other portions of the same organ, and other tissues, always including the spleen, liver and bone marrow, were prepared for histological study. Parasite counts were made for every infection up to the time of sacrificing the chicken.

Biochemical methods.—Normal and infected chickens of 1 to 2 kg body weight were killed by exsanguination. The organs were immediately removed, chilled in ice-cold 0.9% sodium chloride and weighed. Tissue slices were approximately 0.3 mm thick and were prepared as described by Dixon (1943). *Oxygen consumption* and *carbon dioxide evolution* were measured in the Bancroft-Warburg respirometer by the direct method (Dixon, 1943). No retention factor was used in calculating carbon dioxide evolution. For oxygen uptake, about 200 mg wet weight of slices were weighed into 15 ml flasks containing 1.5 ml Krebs phosphate-saline (Krebs and Henseleit, 1932) with 0.01 *M* added glucose and 0.1 ml 20% potassium hydroxide absorbed on a roll of filter paper in the center well. The gas phase was 100% oxygen. For carbon dioxide production, the flasks were filled with 1.5 ml Krebs bicarbonate-saline (Krebs and Henseleit, 1932) containing 0.01 *M* glucose and 0.02 *M* sodium bicarbonate to give a pH of 7.4 in 5% carbon dioxide at 37 C. The gas phase was 5% carbon dioxide and 95% nitrogen. All flasks were equilibrated for 15 minutes at 37 C. and gas exchange was measured for 1 hour. The rates of oxygen uptake and carbon dioxide production of the slice were proportional to the weights taken and remained almost constant during the period of observation.

At the end of each experiment, the *glucose* content of the fluid medium was determined by the method of Nelson (1943)

The *percent dry weight* of each sample of tissue slices was obtained by drying duplicate weighed amounts of freshly-cut slices for 2 hours at 110 C.

From all the measurements just described, the following four Q values were calculated.

1. Q_{O_2} = microliters oxygen consumed in 1 hour by 1 mg dry tissue.

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2. $Q_{CO_2}^{N_2}$ = microliters carbon dioxide liberated under anaerobic conditions from bicarbonate-buffered medium in 1 hour by 1 mg dry tissue. In the tissues of the chicken, this value is approximately equal to the amount of lactic acid formed from glucose and is therefore a measure of the rate of anaerobic glycolysis.

3. $Q_{glucose}^{O_2}$ = microliters glucose consumed under aerobic conditions by 1 mg dry tissue in 1 hour.

4. $Q_{glucose}^{N_2}$ = microliters glucose consumed under anerobic conditions by 1 mg dry tissue in 1 hour.

The *protein index*, a measure of the relative protein content of the tissue slices, was determined by a quantitative biuret reaction. About 200 mg freshly cut slices were homogenized in 2.5 ml 10% trichloroacetic acid. A 1.0 ml aliquot was centrifuged 10 minutes at 500×*g* and the precipitate dissolved in 5 ml 6% sodium hydroxide at 50 C. Then at 20 C, 0.1 ml 20% $CuSO_4 \cdot 10H_2O$ was added. After 60 minutes, the mixture was centrifuged for 10 minutes at 1000×g, and the supernatant liquid was filtered and read against a water blank in the Klett-Summerson photoelectric colorimeter with filter no. KS-54.

$$\text{Protein index} = \frac{\text{colorimeter reading}}{\text{wet weight in mg}} \times 100$$

EXPERIMENTAL RESULTS

Effect of malaria on the organ weights of chickens.—Changes in the organ weights of chickens infected with *P. gallinaceum* and *P. lophurae* are good indicators of the intensity and extent of the tissue changes occurring in the organs during infection. From the total body weight and the organ weight, the percent ratio of organ weight to body weight was calculated. Thus, in normal chickens weighing around 300 g, the spleen weighed 0.59 ± 0.18 g and the liver 11.67 ± 0.18 g. In chickens weighing over 1000 g, the spleen weighed 2.57 ± 0.14 g and the liver weighed 30.45 ± 0.5 g. When the spleen weights as percent body weight of all the chickens were plotted, the ratio was 0.26 ± 0.008 for chickens weighing from 150 to 400 g and was 0.2 ± 0.07 for chickens weighing from 750 to 1800 g, but was higher for chickens weighing 500 to 700 g, with a peak ratio (0.47) reached at about 600 g. The percent ratios of chickens weighing within 500 to 700 g were, therefore, omitted from the following analysis.

The percent ratios of spleen and of liver weight to body weight for normal chickens and for chickens infected with *P. gallinaceum* are given in table 1. Spleen weights were taken from 263 normal chickens and from 563 infected chickens. Liver weights were taken from over half of them. The infected chickens are classified with respect to the stage of parasite used to infect them

TABLE 1.—*Percent organ weight: body weight ± standard error* in chickens infected with* P. gallinaceum.*†

Ratio for normal chickens	Character of infection	Week of infection				
		1st	2nd	3rd	4th–5th	6th or more
0.23±0.006				Spleen		
				Blood-induced		
	A. Light	0.5±0.02*	0.3±0.03*	0.5±0.09*	0.6±0.07*	0.4±0.04
	B. Heavy	0.6±0.02	0.8±0.05	0.9±0.29	0.6±0.01*	0.3±0.02
				Sporozoite-induced		
	C. Light		0.9±0.04	0.6±0.05	0.5±0.07*	0.4±0.02
	D. Heavy	0.5±0.06*	0.9±0.06	0.8±0.05	0.7±0.09	0.4±0.01**
2.51±0.022				Liver		
				Blood-induced		
	A. Light	2.5±0.10*	2.3±0.19*	2.6±0.29*	2.7±0.17*	1.7±0.10
	B. Heavy	3.4±0.18	3.8±0.28	4.0±0.12*	2.4±0.18*	1.8±0.10
				Sporozoite-induced		
	C. Light		3.5±0.10	2.4±0.15*	2.5±0.05**	2.8±0.06
	D. Heavy		3.5±0.17			

* and ** indicate 11 to 20 and <11 in sample, respectively; all other samples varied between 21 and 77.

† Peak number counts were reached during the latter part of the first week in blood-induced infections and during the middle of the second week in sporozoite-induced ones. Lightly infected chickens had peaks of <3 parasites per 10 red cells; heavily infected chickens had peaks of >3 parasites per 10 red cells.

(blood parasites or sporozoites), the intensity of the infection (light or heavy) and the length of the infection (from 1 week to 6 weeks or more). Examination of this table indicates that the percent ratio of spleen weight to body weight doubled the normal ratio (0.23 ± 0.006) during the first week in blood-induced infections and in the heavily infected sporozoite-induced infections (table 1A, B and D). During the second week, it slightly decreased in blood-induced lightly infected birds (1A) but tripled the normal ratio in blood-induced heavily infected chickens and in sporozoite-induced infections. In general, it decreased slowly thereafter (1B, C and D). In general, it decreased slowly for the next 3 weeks. At 6 weeks or more, it was approximately the same in all infections (1A, B, C and D) and was only about 50% larger than the normal value.

The percent ratio of liver weight to body weight could also be correlated with the stage of parasite injected, the intensity and peak of the infection, and the duration of the infection, but the changes were not as large. Thus, the highest ratios (3.5 to 3.8) attained during the 2nd week of infection (1B, C and D) were only about $1\frac{1}{2}$ times the normal ratio (2.38 ± 0.022). These data were compiled from chickens of all weights, as restricted in the first paragraph of this section. The same changes occurred when chickens were separated into 3 weight groups of 100 to 300 g, 800 to 1000 g and over 1000 g. One set of small chickens, however, weighing only 70 g, which had been infected with sporozoites and had an intense blood parasitemia, did not show a marked increase in spleen body weight ratio during the 2nd week (0.48 ± 0.01). This fact may indicate that the spleen is not as reactive in very young as in older chickens and may account for Terzian's (1941) negative results in small chickens infected with *P. lophurae*.

The changes in percent ratio of spleen weight to body weight were not pronounced during infection with *P. lophurae*, and normal values were again prevalent 6 weeks after infection. At or slightly after the peak of the infection, the highest ratio was attained and generally was about twice the normal value.

The percent ratios of the lung, kidney and heart to body weight showed no change during infection with either *P. gallinaceum* or *P. lophurae*.

Effect of malaria on the glucose metabolism of chicken tissues.—Since spleens of chickens infected with *P. gallinaceum* showed the greatest percent increase in weight and the greatest evidence of connective tissue alteration of all the organs studied, they were subjected to the most extensive biochemical investigation. Figure 1 records the observations on spleen slices from 12 normal chickens and 36 chickens infected with blood stages of *P. gallinaceum*. The infected birds were divided into 4 groups according to the stage of infection at the time the spleens were removed for slicing:

1. The acute initial rise in parasitemia, divided into 4 subgroups of progressively increasing parasitemia.

2. The beginning crisis, marked by the appearance of crisis forms and representing the approximate peak of parasitemia.

3. The crisis, as indicated by an undoubted drop in parasitemia and the presence of degenerate crisis parasites.

4. The developed infection, characterized by a low grade, yet persistent, parasitemia.

The mean parasitemia for the different groups of infected chickens is shown in figure 1. The progressive and regressive histological changes during the rise and fall of the infection have

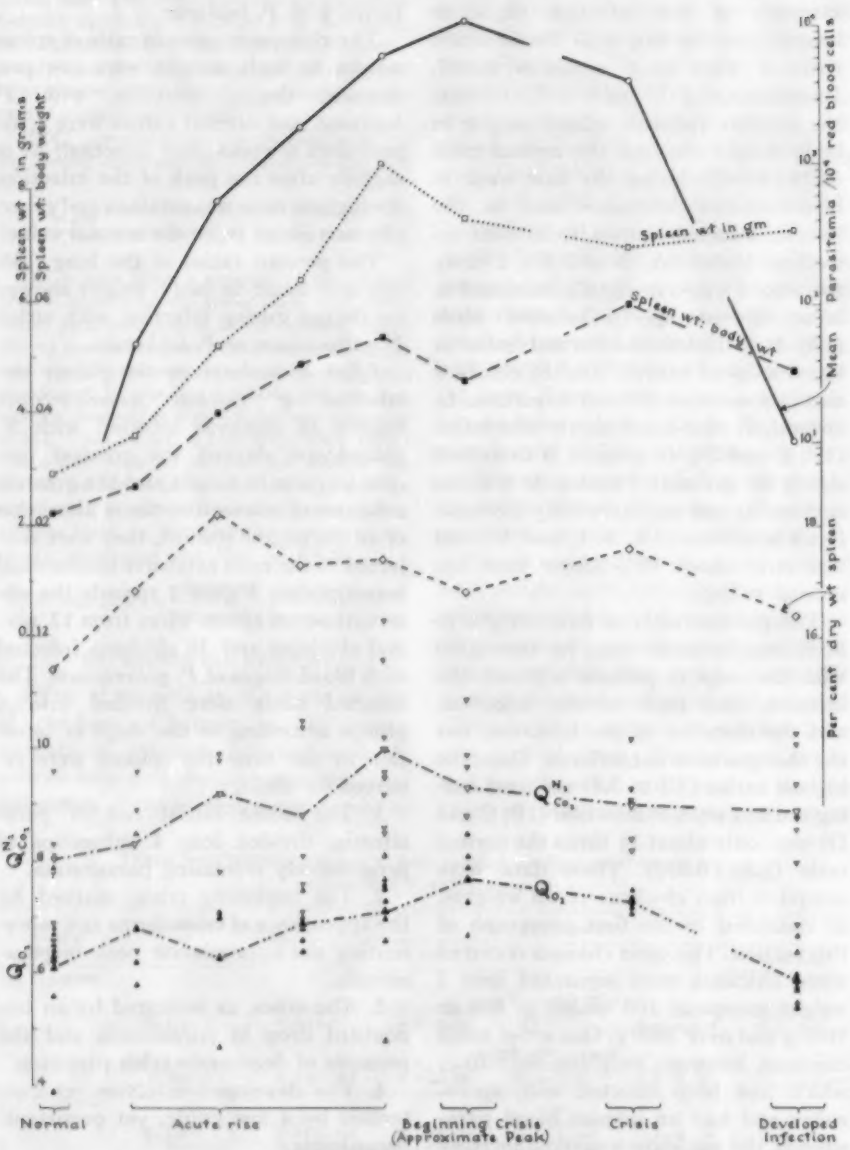

FIG. 1—The glucose metabolism of spleen slices from chickens at different stages of infection with *P. gallinaceum*. The spleen weight: body weight ratio is given in percent.

already been described in Part I of this series. The associated splenomegaly is clearly indicated by the plot of spleen weight and percent ratio spleen weight to body weight against the stage of infection (fig. 1).

In spite of the great changes in size and cellular activities of the spleen, such as phagocytosis and mitosis, during infection with *P. gallinaceum*, there was no statistically significant difference in the percent dry weight, the rate of oxygen consumption (Q_{O_2}) or the rate of anaerobic glycolysis ($Q^{N_2}_{CO_2}$) between normal and infected spleens (fig. 1).

In a second smaller group of spleen slices from normal and infected chickens, measurements of the rate of glucose utilization under aerobic ($Q^{O_2}_{glucose}$) and anaerobic ($Q^{N_2}_{glucose}$) conditions and of the relative protein content (protein index) were made in addition to those recorded in figure 1. Again, the results with slices from normal and from infected spleens were almost identical (table 2).

There was also no difference between the Q_{O_2} and $Q^{N_2}_{CO_2}$ of slices from the livers, hearts, kidneys and lungs of normal chickens and those infected with *P. gallinaceum*.

TABLE 2.—*Effect of infection with* P. gallinaceum *on the glucose metabolism of chicken spleen slices.*

Measurement	Normal spleen	Infected spleen
Spleen weight in grams	2.1	4.9
Percent dry weight of spleen	13.4	15.5
Protein index	112.0	133.0
Q_{O_2}	9.0	8.2
$Q^{N_2}_{CO_2}$	8.2	8.8
$Q^{O_2}_{glucose}$	2.7	2.7
$Q^{N_2}_{glucose}$	4.7	4.7

The values given above are the means of observations on 4 normal and 6 infected chickens. The infected group was made up of 2 chickens from each of the following stages of infection: acute rise, crisis and developed infection.

DISCUSSION

The unchanging percent dry weight and protein content of chicken spleen during infection with *P. gallinaceum* indicate that the enlargement of the spleen is a true increase in the solid mass of the organ and not merely an increase in fluid content. The constancy of the rates of aerobic and aerobic glucose metabolism per gram of splenic tissue suggests that the cellular hyperplasia involves the formation of new cells with the same glucose metabolism as the cells originally present in the uninfected spleen. Dying lymphocytes and phagocytes must be rapidly destroyed and removed after death, for their persistence would tend to lower the metabolic rate per gram of spleen. Rapid removal of dead cells is consistent with the histological finding reported in Part I.

A possible exception to these general conclusions may occur at the time of the intense immune reaction of the crisis. At this stage of infection, swollen macrophages and degenerating lymphocytes persisted and marked increases in intracellular fluid occurred—conditions which would be expected to reduce the total number of viable cells per unit mass of spleen. Therefore, at the crisis, the rate of glucose metabolism per living cell may increase although the rate per gram of spleen remains constant.

It should not be concluded from these results that all changes in connective tissue are unaccompanied by metabolic alterations. For example, the consolidation of mouse lung produced by either infection with murine pneumonitis virus or by intranasal instillation of egg yolk resulted in a lowering of oxygen consumption and a raising of the anaerobic glycolysis (Moulder and Weiss, 1951).

SUMMARY

Infection of chickens with *Plas-*

modium gallinaceum produced a marked enlargement of the spleen and, to a lesser extent, the liver. Lung, kidney and heart were unaffected. Infection with *Plasmodium lophurae* also resulted in splenomegaly, but the increase in organ size was less than in infections with *P. gallinaceum*.

Spleen splices from chickens infected with *P. gallinaceum* exhibited normal percent dry weights, protein contents, and rates of aerobic and anaerobic glucose metabolism throughout the entire course of infection. Slices from liver, heart, lung and kidney were also indistinguishable from normal slices in these respects.

It is concluded that enlargement of the chicken spleen during infection with *P. gallinaceum* is a true increase in living tissue and that cellular hyperplasia involves the formation of new cells with the same glucose metabolism as the original cells of the uninfected spleen.

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PROTEOLYTIC ACTIVITIES OF CERTAIN MEMBERS OF THE GENUS *CORYNEBACTERIUM**

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With the exception of *Corynebacterium diphtheriae* relatively little attention has been given to the physiology of other members of the genus *Corynebacterium*. Many species are important as plant and animal pathogens. The most important species, from the viewpoint of veterinary medicine, *Corynebacterium pyogenes*, has been reported to cause the liquification of Loeffler's blood serum slants (Merchant, 1935). Since the proteolytic activities of an organism aid in its classification and, in the case of pathogens, may be related to toxigenicity, further information regarding the protein digesting ability of the corynebacteria seemed desirable. This investigation was undertaken to study the ability of 124 strains of the genus *Corynebacterium* to digest selected animal protein substrates.

EXPERIMENTAL METHODS

Since the proteolytic activities of an organism may depend upon the substrate present, seven different protein mediums were used. The litmus milk and gelatin hydrolysis tests were conducted in test tubes, while the other substrates were added to agar plates. Sterile solutions of albumin and γ globulin were added to sterile stock culture agar base (Difco). This mixture was heated carefully in a boiling water bath until a fine homogenous suspension of heat-denatured protein appeared. The medium was then cooled to 45 C and plates poured. The other proteins were not readily coagulated by heat and were added to the medium prior to autoclaving. The various protein substrates studied, their source and method of sterilization are shown in table 1.

The cultures were obtained from the collection of the Department of Bacteriology, Kansas State College, and from several laboratories throughout the United States.‡ Stock cultures maintained on stock-culture agar slants were used as sources of inoculum for the protein mediums. The cultures of known plant pathogens were incubated at 30 C and all other strains at 37 C. The tests for protein hydrolysis were read after 5 days growth. Plates containing the proteins still in solution were flooded with 5% acetic acid to precipitate the undigested protein. Hydrolysis of the protein was indicated by a distinct, clear zone around the colonies.

TABLE 1.—*Sources of protein substrates and method of sterilization.*

Protein substrate	Source	Method of sterilization
Egg albumin	Fresh hen's egg	Aspetic removal from egg and sterility tested
Bovine γ globulin	Armour	Filtration
Casein	Difco	Autoclaving in medium
Lactalbumin	Nutritional Biochemicals Co.	Autoclaving in medium
Lactoglobulin	Pentex, Inc.	Autoclaving in medium
Litmus milk	Difco	Autoclaving in medium
Gelatin	Difco	Autoclaving in medium

RESULTS AND DISCUSSION

In table 2 is recorded the presence or absence of proteolysis on 7 protein-containing mediums by 72 strains, representing 13 species, of the genus *Corynebacterium*. Only *C. pyogenes* (31

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TABLE 2.—*Hydrolysis of various proteins by different species of* Corynebacterium.

	*Litmus milk	Number of strains	Gelatin	Egg albumin	Bovine serum γ globulin	Casein	Lactal-bumin	Lacto-globulin
C. pyogenes	C.R.D.	31	+	+	+	+	+	+
C. renale	D	7	−	−	−	+	−	−
C. xerosis	N.C.	4	−	−	−	−	−	−
C. equi	N.C.	5	−	−	−	−	−	−
C. pseudodiphthericum	N.C.	7	−	−	−	−	−	−
C. pseudotuberculosis	N.C.	9	−	−	−	−	−	−
C. Ulcerans	N.C.	1	−	−	−	−	−	−
C. fascians	N.C.	2	−	−	−	−	−	−
C. sepedonicum	N.C.	1	−	−	−	−	−	−
C. tumescens	C.R.D.	1	−	−	−	+	+	−
C. helvolum	D	1	+	+	+	+	+	+
C. poinsettiae	N.C.	1	+	−	−	+	−	−
	N.C.	1	−	−	−	+	−	−
C. rathayi	N.C.	1	−	−	−	−	−	−

* C. = coagulation; D. = digestion; N.C. = no change; R. = reduction.

strains) and *C. helvolum* caused digestion of all 7 substrates. *C. pyogenes* gave complete reduction and digestion of litmus milk, while the growth of *C. helvolum* caused digestion only. *C. tumescens*, *C. poinsettiae* and *C. renale* showed proteolytic activity limited to certain of the substrates. The remaining 8 species failed to digest any of the proteins.

Of special significance is the comparison of the protein digesting abilities of *C. pyogenes* and *C. renale*, both of which are important pathogens for domestic animals. The data shown in table 2 indicate that these two species could probably be differentiated on the basis of their proteolytic activities on substrates other than Loeffler's blood serum and litmus milk. Although difficulties were encountered in the preparation of satisfactory poured plates using wheat gluten, the results indicated that of all the animal pathogens studied, only *C. pyogenes* could digest this protein.

The data in table 3 indicate the proteolytic activity of 52 unclassified strains of the genus *Corynebacterium*. None of the 15 strains isolated from milk showed any proteolysis of the 7 substrates, including limus milk and three separate components of the milk proteins, namely, casein, lactalbumin, and lactoglobulin. It is of interest that the single strain isolated from eggs utilized all substrates except egg albumin. Only three strains isolated from human sources showed proteolytic activity. These data indicate that proteinase production is limited to relatively few species of the genus *Corynebacterium*. It should be emphasized that all proteins employed in these experiments were subjected to heat treatment at some point in the preparation of the mediums. Thus, all of the results reported in tables 2 and 3 represent the production of proteinases by organisms growing on wholly or partially denatured protein substrates.

TABLE 3.—*Hydrolysis of different proteins by various unclassified strains of corynebacteria.*

Source	Litmus milk	Number of strains	Gelatin	Egg albumin	Bovine serum γ globulin	Casein	Latcal-bumin	Lacto-globulin
Milk	N.C.	15	−	−	−	−	−	−
Egg	N.C.	1	+	−	+	+	+	+
Human (throat)	N.C.	17	−	−	−	−	−	−
	D	1	+	−	+	+	+	+
	N.C.	1	+	−	−	−	−	−
Human (vagina)	N.C.	16	−	−	−	−	−	−
	N.C.	1	−	−	−	+	−	−

Earlier studies of proteolytic enzymes showed that native proteins are not hydrolyzed unless some simpler source of nitrogen is available to the organisms (Rettger et al, 1916). To investigate this phenomenon in relation to the corynebacteria, poured plates were prepared using sterilized agar mediums to which were added protein solutions sterilized by filtration. The proteins tested were lactoglobulin and egg albumin. Although typical growth occurred, in no instance did any of the 124 strains show digestion of the undenatured substrate. Hence, the proteolytic activities of these organisms seem to be limited to denatured protein molecules.

SUMMARY

Using the poured plate technique, 124 strains of the genus *Corynebacterium* were tested for their ability to digest litmus milk, gelatin, egg albumin, bovine serum γ globulin, casein, lactalbumin and lactoglobulin. All 31 strains of *C. pyogenes* and the single strain of *C. helvolum* hydrolyzed all 7 substrates. *C. renale* (7 strains) digested only casein. A single strain of *C. tumescens*, 2 strains of *C. poinsettiae*, and 4 of 52 unidentified strains digested one or more of the 7 substrates. The remaining 30 identified strains, distributed among 8 species, and 48 unidentified strains failed to digest any protein tested. None of the organisms studied could digest lactoglobulin, or egg albumin in the native state, even in the presence of simpler nitrogenous compounds.

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ERYTHROMYCIN IN EXPERIMENTAL LEPTOSPIROSIS

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There are numerous conflicting clinical studies of the treatment of leptospirosis by antibiotics,[1] but studies of the treatment of the experimental disease have emphasized that treatment must be commenced early to be of value.[2] This would be expected from the nature of the infection. A recent report[3] of antibiotic treatment of chick embryos infected with *Leptospira icterohaemorrhagiae* suggested that erythromycin might be effective. This present study deals with the sensitivity in vitro of leptospiras to erythromycin and the treatment with this antibiotic of experimental Weil's disease in young guinea pigs (150 to 200 g) and young hamsters (45 to 50 g).

Sensitivity of leptospira strains to erythromycin

Strains of pathogenic leptospiras (kindly supplied by Dr. J. C. Broom from the Leptospira Type Culture Collection, Wellcome Laboratories of Tropical Medicine, London) maintained at 30 C in a modified Korthof medium containing 10% rabbit-serum were tested against various concentrations of erythromycin. Organisms were counted directly under dark-ground illumination using 0.01 ml culture under a three-fourth inch coverslip of known area and a graticule eyepiece. This method is accurate to approximately 10% and may be made sensitive enough to detect approximately 100 organisms/ml.

A preliminary test was carried out to determine the effect of the size of inoculum and optimum time for testing the end point. Dilutions of erythromycin in 1 ml Korthof medium were inoculated with *Lept. icterohaemorrhagiae* (Jackson) to a concentration of 10^4, 10^5, and 10^6 organisms per ml. Table 1 shows that, although there is a variation in end point after 4 days incubation, both bacteriostatic and bactericidal levels are clear-cut after 10 days. Cultures from the tubes where no spirochetes were seen failed to grow. The size of the inoculum, therefore, was unimportant, when all determinations of sensitivity were read after 10 days incubation, during which time the erythromycin was shown to be stable and not utilized.

For in vitro tests of sensitivity, 10-fold dilutions of erythromycin from 1.0 μg/ml to 0.00001 μg/ml were set up in modified Korthof's medium, with control medium without antibiotic. One ml volumes were inoculated with approximately 10^6 leptospiras in 0.05 to 0.1 ml of a 7- to 10-day culture grown at 30 C in the same medium.

Sensitivities of various leptospira strains, which in the given concentration of erythromycin were sterilized in 10 days, were as follows:

Sensitive to 0.001 μg/ml erythromycin: Lept. canicola (Utrecht); *Lept. grippotyphosa* (Moscow V); *Lept. mitis* (Johnson); *Lept. sejroe* (M 84).

Sensitive to 0.001 to 0.01 μg/ml; Lept. icterohaemorrhagiae (Field); *Lept. icterohaemorrhagiae* (Jackson); *Lept. canicola* (Aldgate); *Lept. australis A; Lept. bataviae* (62); *Lept. hebdomadis.*

Sensitive to 0.01 μg/ml: Lept. pomona.

Addition of penicillin in sub-bactericidal concentrations (0.001 u./ml) or bactericidal concentrations (0.01 u/ml) with erythromycin showed neither synergism nor antagonism between the antibiotics, using the Jackson strain of *Lept. icterohaemorrhagiae* as the test organism.

Determination of erythromycin in serum and tissues

The vertical diffusion method was used for the determination of erythromycin in serum and tis-

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* Nuffield Dominions Demonstrator.

1. Valentine, F. C. O. and Shooter, R. A. 1954, Recent Advances in Chemotherapy. III. Antibiotics, J. and A. Churchill, p. 263.
2. Uhlenhuth, P. and Schoenherr, K. 1951, Ztschr. f. Immunitätsforsch. u. exper. Therap. **108**: 289–300.
3. Ormsbee, R. A. 1953, Proc. Soc. Exper. Biol. & Med. **83**: 815–816.

TABLE 1.—*Effect of inoculum size and duration of incubation on sensitivity of* Lept. icterohaemorrhagiae (*Jackson*) *to erythromycin.*

Duration of incubation (days)	Original inoculum (millions per ml)	Count in millions of leptospiras/ml with erythromycin (μg/ml)					
		0	0.0001	0.001	0.01	0.1	1.0
4	0.01	2.6	1.0	0.0002	0.0003	0.0002	0
4	0.1	9.0	7.4	1.2	0.2	0	0
4	1.0	102	40.6	2.2	2.0	0.3	0.0002
10	0.01	159	156	93	0.08	0	0
10	0.1	120	81.7	157	0.16	0	0
10	1.0	66.7	41.4	35.3	6.4	0	0

sues. A medium based on Difco streptomycin-assay agar was used, as follows:

Beef extract (Lemco)	0.5 g
Yeast autolysate (Glenochil)	7.0 ml
Bacto peptone	3.0 g
Bacto agar	4.5 g
Distilled water	300 ml

pH adjusted to 8.0; autoclave.

Ten percent normal guinea pig serum was then added to the medium which was kept melted at 45 C while an overnight broth culture of a stock laboratory strain of α-haemolytic streptococcus was added to a final 10^{-4} dilution. Tubes of 3 mm diameter were filled to 4 cm depth with the melted inoculated medium.

After solidification the inoculated medium was overlaid with about 1 cm depth of solutions containing 0.001 to 0.12 μg/ml pure erythromycin in 10% normal guinea pig serum in Sørensen's *M*/15 phosphate buffer, pH 8.0. After 24 hours at 37 C the length of the zone of inhibition of growth was measured with a low power (x 22 approximately) travelling microscope and vernier scale. Using the average of 4 replicates for each erythromycin concentration, a standard reference curve of erythromycin concentration: length of zone of growth-inhibition was prepared.

Test serums containing unknown amounts of erythromycin were diluted 1:10 in phosphate buffer and overlaid upon the inoculated medium, incubated, and the zones of inhibition measured as above. Erythromycin concentrations were read from the reference curve. This method enables erythromycin concentrations as low as 0.01 μg/ml to be determined, using as little as 0.1 to 0.2 ml serum easily obtainable from guinea pig ear vein blood. Serums with high antibiotic concentrations may be diluted to give readings within the optimum range of the reference curve.

To measure tissue concentrations, weighed samples of the fresh tissues were ground with a known volume of buffer, and an aliquot of the supernatant of the suspension treated, after suitable dilution, as described above for serum.

Table 2 shows concentrations of erythromycin in tissues taken 1 to 2 hours after at least the third dose, from animals treated twice daily with various doses orally or intraperitoneally. Serum concentrations after intraperitoneal injection are approximately 10 times those after equivalent oral dosage.

Treatment of experimental infections

Young guinea pigs were infected intraperitoneally with 1 to 2 ml of a saline-liver suspension containing approximately 100 million leptospiras prepared from a guinea pig freshly dead or dying of infection with the Field strain of *Lept. icterohaemorrhagiae* maintained at maximum virulence by continuous guinea pig passage. The picture of leptospirosis in these guinea pigs was unmistakable. Fever, hemorrhage, jaundice, uraemia and leptospiremia were classical. Death occurred inevitably in 4 to 5 days in untreated animals.

After it appeared that erythromycin was toxic for guinea pigs, hamsters were infected as for guinea pigs. Again the picture of leptospirosis was classical.

TABLE 2.—*Tissue concentrations of erythromycin in guinea-pigs.*

Dose (twice daily), mg erythromycin/kg	Route	Erythromycin concentration in:		
		Serum (μg/ml)	Liver (μg/gm)	Kidney (μg/gm)
16	Oral	0.01–0.02		
32	Oral	0.02–0.08		
53	Oral	0.2		
62*	Oral	0.1–0.2	0.9	0.4
333	Oral	1.7	50	
		2.4	72	
		4.8	87	
16	Intraperitoneal	0.3	16	
62*		2		

* Average of almost identical values from 4 animals in these groups.

TABLE 3.—*Treatment with oral erythromycin of experimental leptospirosis in guinea pigs.*

Treatment			Number of animals		Days after treatment commenced on which deaths occurred*											
Started (days post-infection)	Dose (mg/kg)	Duration (days)	Used	Survived	3	4	5	6	7	8	9	10	11	12	13	14 or over
1	110	6	4	2					*1*		*1*					
	65	3	4	4												
	33	3	5	2						*1*	*1*					1
	16	5	10	6					*1*	*1*	*1*	*1*				
2	110	5	5	1					*1*		*1*	*2*				
	65	3	5	1						*1*	*1*	*1*		*1*		
	33	3	5	2							*2*	*1*				
	16	4	10	3					*1*	*2*	*1*			*1*	*2*	
3	110	4	4	0							*1*	*3*				
	65	3	5	0					*2*	*1*	*2*					
	33	3	5	0		4	1									
	16	3	10	0	1	8	1									
Untreated controls			8	0			7	1								
			10	0		2	8									
			5	0		5										
			10	0		4	6									
Uninfected controls																
	110	6	5	1					*1*		*2*			*1*		
	65	3	5	3		*1*			*1*							
	33	3	5	5												
	16	6	5	5												

* Numerals in roman type refer to deaths from leptospirosis.
Numerals in italic type refer to deaths from erythromycin toxicity.

At various intervals after inoculation groups of infected animals were treated orally or by injection with doses of erythromycin calculated to produce blood concentrations exceeding bactericidal levels measured in vitro (0.01 μg/ml for the strain used). The results of treatment, with the fate of control untreated and uninfected groups, are shown in tables 3 and 4. Defervescence occurred within 24 hours of commencing treatment. In another experiment treatment with 60 mg erythromycin/kg in two doses for one day only, started either the first or second day post-infection, prolonged the life of 5 animals in each group 2 to 3 days after the controls died, although all died of typical leptospirosis eventually.

It will be seen, from tables 3 and 4, that many infected animals died without signs of leptospirosis after apparent

TABLE 4.—*Treatment with intraperitoneal erythromycin of experimental leptospirosis in guinea pigs and hamsters.*

Animal	Group	Treatment			Number of animals		Days after treatment commenced on which deaths occurred*									
		Started (days post-infection)	Dose (mg/kg)	Duration (days)	Used	Survived	4	5	6	7	8	9	10	11	12	13 or over
Guinea pig		1	33	3	5	0				*1*		*3*		*1*		
		2	33	3	5	0						*2*	*3*			
		3	33	3	5	0	4	1								
		Untreated controls			5	0	5									
		Uninfected controls														
			33	3	5	0				*1*	*1*	*3*				
Hamster	A	1	60	6	5	5										
	B	1	30	6	5	1					*1*			*1*	*2*	
	C	3	60	6	4	1	1	2								
	D	3	30	6	5	0	1	1		1		*1*	*1*			
	E	Untreated controls			5	0	1	4								
	F	Uninfected controls	60	6	5	2										*3*
	G		30	6	5	0										*5*

* Numerals in roman type refer to deaths from leptospirosis.
Numerals in italic type refer to deaths from erythromycin toxicity.

recovery from the infection. Leptospiras could not be found in these animals. Their lesions were either healed, or minimal and regressing. Many uninfected control animals also died after erythromycin treatment. Death was attributed to erythromycin toxicity. Animals became listless, emaciated but afebrile, sometimes developed diarrhea and died from the 3rd to 12th (average 7th) day after treatment commenced. Some hamsters died after an average of 13 days after commencement of treatment. The cause of this toxicity, which is the subject of a separate communication, is unknown.[4] Animals dying, in our opinion, not from leptospirosis but from toxicity are indicated in italic numerals in the tables.

The carrier state and residual renal lesions

A chronic renal carrier state is a well known feature of leptospirosis in animals, especially after unsatisfactory antibiotic treatment.[5] Kidneys of 17 survivors of experiments recorded in table 3, sacrificed 15 to 45 days after infection, were examined for leptospiras by culture, microscopically by dark-ground and silver-impregnation stainings and by guinea pig inoculation. Three were found to be carriers. All 17 had agglutinin titers to *Lept. icterohaemorrhagiae* (formolized culture) ranging from 1:300 to 1:2,500, with 1:20,000 accompanied by a blood urea level of 120 mg/100 ml in one case. Two other animals, one of them found to be a carrier, had abnormally high blood urea levels of 60 and 90 mg/100 ml. Sections of kidneys from these survivors showed occasionally slight scarring, but no gross lesions even in the carriers.

4. Kaipainen, W. J. and Faine, S. 1954, Nature, **174**: 969–970.

5. Brunner, K. T. and Meyer, K. F. 1949, Proc. Soc. Exper. Biol. & Med. **70**: 450–452.

TABLE 5.—*Renal carriers after treatment. Results of guinea pig inoculation with kidney emulsion from guinea pigs treated orally with erythromycin from the first day postinfection.*

Treatment		Number of animals	Day after infection of		Number of renal carriers
Duration (days)	Dose (mg/kg)		Death	Sacrifice	
6	64	4		10	1
	32	5		11	0
3	64	3	9		0
		2		10	2
	32	3	8, 9, 10		3
		1		10	1

One guinea pig which had apparently recovered with treatment unexpectedly developed typical leptospirosis on the 13th day after infection, and died, presumably from reinfection from a renal focus. The organism isolated from this guinea pig was as sensitive to erythromycin as the parent strain, as were leptospiras isolated from the other carriers.

Table 5 shows that in an experiment to test the duration of antibiotic treatment necessary to abolish the carrier state 6 days therapy was more effective than 3 days in reducing the proportion of carriers by the 11th day after infection. Animals which did not die of leptospirosis within 3 weeks of inoculation with kidney emulsion from suspected carriers did not show antibodies against *Lept. icterohaemorrhagiae* when tested at the end of this time.

DISCUSSION

The sensitivity in vitro of leptospiras to erythromycin was independent of the size of the inoculum. These experiments are not strictly comparable with those of Borg-Petersen and Schmidt,[6] who found that the end point for penicillin-

6. Borg-Petersen, C. and Schmidt, M. R. 1945, Acta path. et microbiol. Scandinav. **62**: 462–474.

sensitivity determinations was different for "thin" (young) and fully-grown cultures.

Although the strains of leptospiras tested were susceptible to blood and tissue levels of erythromycin easily attainable by oral or parenteral dosage, treatment of experimental Weil's disease in this work has been complicated by the unexpected toxicity of erythromycin for guinea pigs. We have been unable to find reference to this toxicity in the literature on erythromycin.[7] In this connection the peculiar susceptibility of the guinea pig to penicillin may be recalled. The nature or cause of the toxicity in either case is unknown.

In the experiments there is a clear-cut clinical and temporal difference between death of the guinea pigs from leptospirosis, generally on the 4th, 5th or 6th day and from erythromycin on the 6th or 7th day or later, usually after a period of apparent complete recovery from the infection. Some borderline cases may have succumbed to the combined effects of the infection and its treatment where they might just have survived had the antibiotic not been toxic.

Unfortunately, the guinea pig is the most suitable experimental animal for Weil's disease, although hamsters may also be used. In one experiment with hamsters, however, the results anticipated from the guinea pig experiments were confirmed. Early adequate erythromycin treatment (30 to 60 mg/kg for 6 days) cured leptospirosis, leaving only one carrier among 11 survivors.

Guinea pig kidneys were found to contain 2 to 4 times the erythromycin concentration of the serum (table 2). Renal carriers after adequate erythromycin treatment were rare. This is in contrast to the carrier rate with penicillin treatment described by other workers.[8] Residual lesions were also unimportant, corresponding to similar experiments with terramycin, where scarring only was found.[8]

The nature of severe leptospirosis is such that the course of the infection may become irreversible, about the third day in these experimental infections. This stage corresponds to the appearance of severe hemorrhage, onset of jaundice and anemia, and gross leptospiremia. It has already been observed in experimental treatment with terramycin and aureomycin[2] that early treatment is essential to be of value. On the other hand, the fact that treatment for only a single day prolonged life for 2 to 3 days suggests that the antibiotic causes a rapid but incomplete reduction in the number of organisms in the animal. The use of very large doses in the later stages was obviated by toxicity in these experiments, but no antibiotic alone could reverse rapidly the severe renal damage to be found late in the jaundiced stage.

It is difficult and dangerous to argue too far from the analogy between the naturally-occurring infection and experimental Weil's disease in guinea pigs. Technical difficulties make trials of antibiotic treatment in experimental forms of the clinically milder leptospiroses difficult to do and more difficult to evaluate. The fact that an early enough diagnosis may not be made in severe human or animal infections means that in any case antibiotic treatment may always be of limited value in practice.

SUMMARY AND CONCLUSIONS

1. Various pathogenic leptospiras are sensitive to concentrations of ery-

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8. Gädeke, R. and Schoenherr, K. 1952, Ztschr. f. Immunitätsforsch. u. exper. Therap. **109**: 488–502.

thromycin attainable in blood and tissues.

2. Early adequate treatment with erythromycin cured experimental infection with *Leptospira icterohaemorrhagiae* in guinea pigs and hamsters with a low carrier rate and without residual lesions.

3. The toxicity of erythromycin for guinea pigs and hamsters may obscure the results of treatment of experimental infection in these animals.

THE EFFECT OF CORTISONE ON *PLASMODIUM BERGHEI* INFECTIONS IN THE WHITE RAT

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The course of infection with *Plasmodium berghei* in the white mouse is markedly altered by cortisone treatment of the host.[1] Cortisone alters the state of the host's erythropoietic system and causes a decrease in the number of reticulocytes, the host cells preferentially parasitized by *P. berghei*. The increased survival time for infected mice treated with cortisone has been directly related to the decrease in reticulocytes available to the parasite.

Although blood-induced infections with *P. berghei* in the white mouse are invariably fatal, those induced in mature white rats are not. The purpose of this investigation was to ascertain whether *P. berghei* infections in the white rat respond to cortisone treatment in a similar or different way than do infections in the white mouse.

MATERIALS AND METHODS

The hosts were a single strain of young, 60 gram, white female rats (Holzman). The KBG 173 strain of *P. berghei*, which was utilized for all infections, had been maintained in this laboratory for two years by biweekly passage in white rats via the intraperitoneal route. Both experimental and stock animals have been kept in an air conditioned room at a temperature of approximately 72 F, with alternating periods of 12 hours of light and 12 hours of darkness.

Fourteen animals were divided into four groups. The four animals whose course of infection is shown in figure 1 were infected with *P. berghei* but were not treated with cortisone. The course of infection shown in figure 2 is derived from four animals which received a total of 20 mg cortisone acetate, which was administered intramuscularly in four equal doses over a period of five days. Two doses were given on the two days preceding infection with *P. berghei*, and the remaining two doses were given on the two days following the day of infection. Each infection was initiated by inoculating into the tail vein 0.2 ml citrated blood which contained approximately 14 million parasites. The three animals whose condition is shown in figure 3 were given a total of 20 mg cortisone acetate in four equal doses over a period of five days, but were not infected with *P. berghei*. Three uninfected animals not treated with cortisone, ie., normal controls, defined the normal range for total red blood cell counts and reticulocyte counts which are shown as shaded areas on figures 1, 2, and 3.

The course of malarial infection and the effect of cortisone were followed in the host's blood by daily blood smears for parasite and reticulocyte counts, and total red blood cell counts every second day. All blood for these counts was obtained from the tail vein. Blood smears for reticulocytes were made on glass slides coated with dry films of brilliant cresyl blue (3% in 95% ethyl alcohol). The smears were then placed in a moist chamber for 5 to 10 minutes and on removal were rapidly dried and examined within 24 hours. Counts are expressed as reticulocytes per ten thousand red blood cells. Parasite counts were made according to the method of Taliaferro and Taliaferro[2] and are expressed as the number of parasites per ten thousand red blood cells. Total red blood cell counts are expressed as red cells per cubic millimeter of blood.

EXPERIMENTAL

Reticulocyte counts in normal control animals vary from 500 to 1700, a difference of 1200 per ten thousand red blood cells. Total red blood cell

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2. Taliaferro, W. H. and Taliaferro, L. G. 1940, J. Infect Dis. **66**: 153–165.

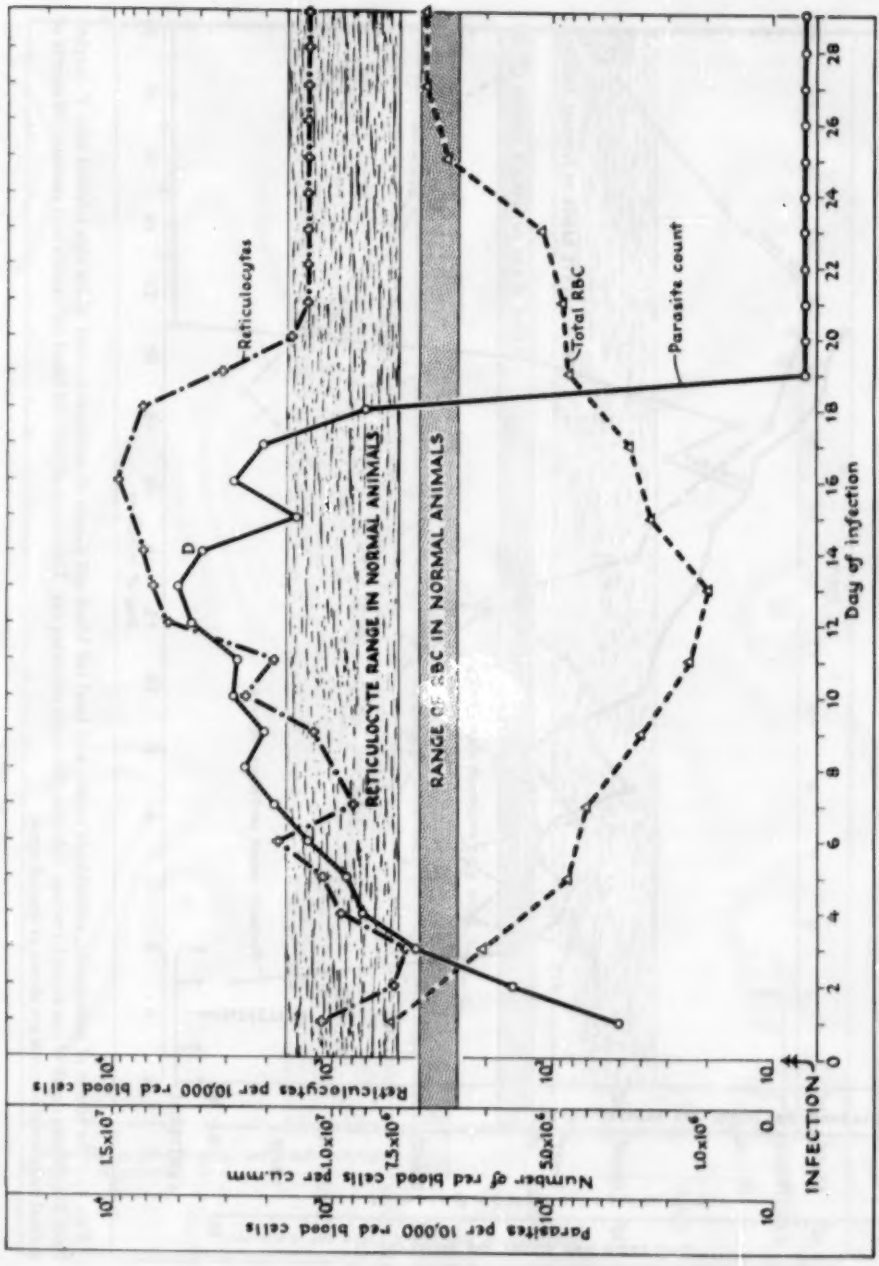

FIG. 1.—The course of parasitemia, reticulocyte counts and total red blood cell counts of normal white rats infected with *P. berghei*. D indicates death of one animal. The ranges of total red blood cell counts and reticulocyte counts of normal uninfected controls are shown as shaded areas.

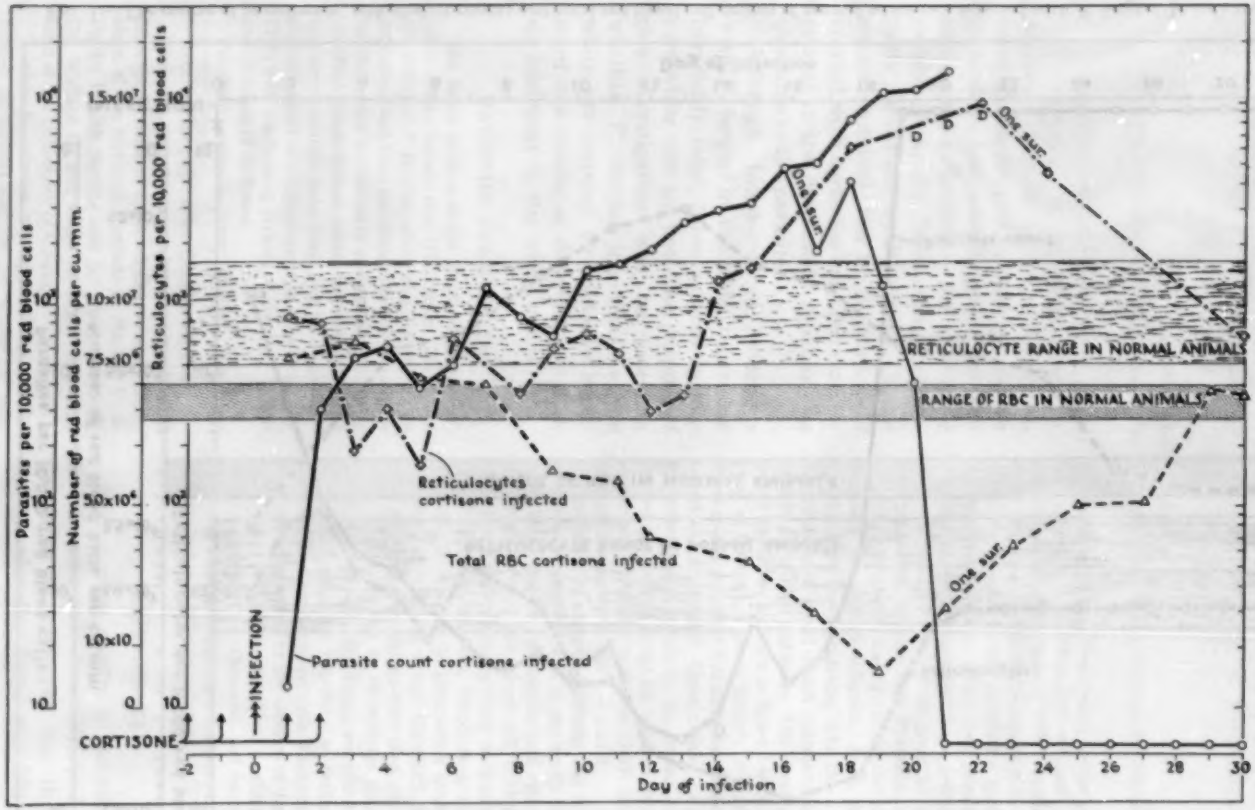

FIG. 2.—The course of parasitemia, reticulocyte counts and total red blood cell counts of cortisone-treated white rats infected with *P. berghei*. Each D indicates death of one animal; one sur. indicates the single surviving rat. The ranges of total red blood cell counts and reticulocyte counts of normal uninfected controls are shown as shaded areas.

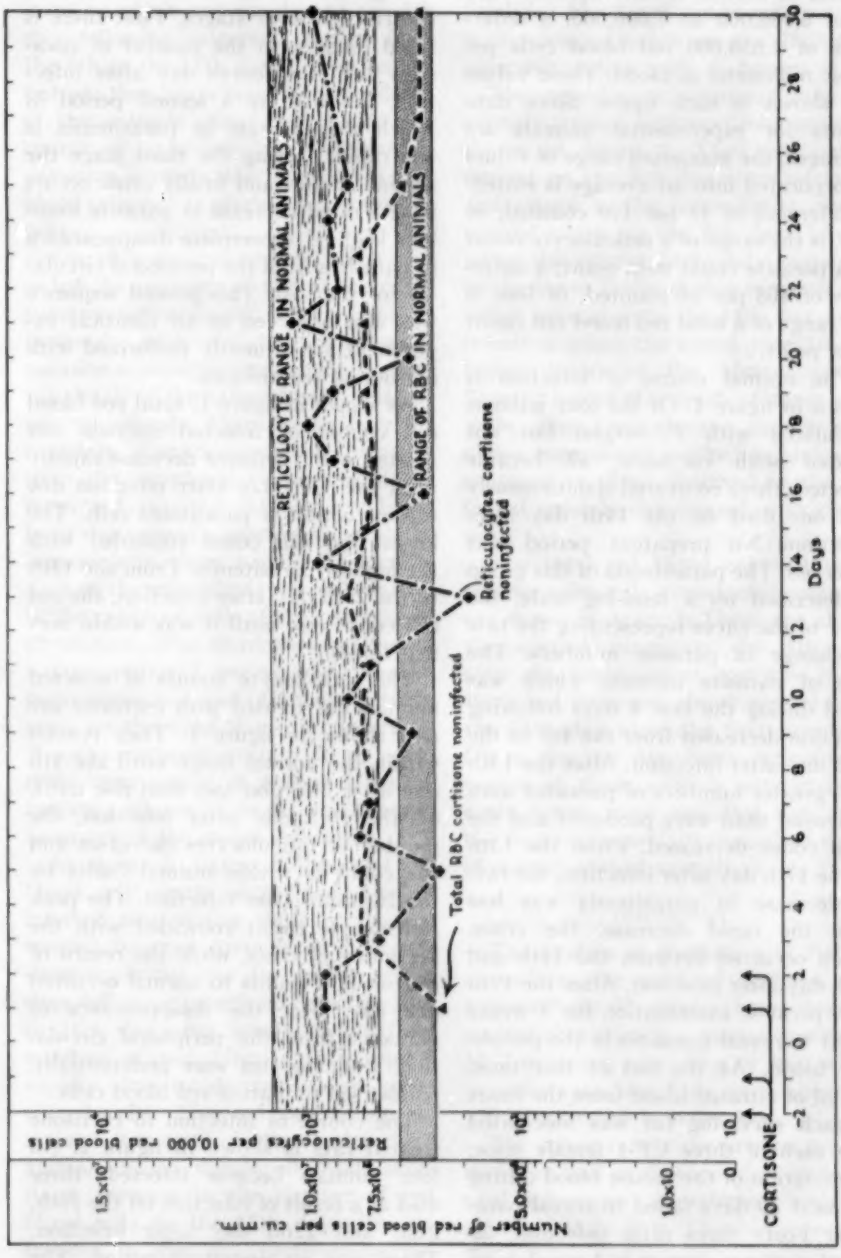

FIG. 3.—Total red blood cell and reticulocyte counts of uninfected rats treated with cortisone. The ranges of total red blood cell counts and reticulocyte counts of normal uninfected controls are shown as shaded areas.

counts in normal control animals vary from 6,350,000 to 7,000,000, a difference of 1,650,000 red blood cells per cubic millimeter of blood. These values are shown in each figure. Since data points for experimental animals are averages, the maximum range of values incorporated into an average is stated: a difference of 15 per 100 counted, or less, is the range of a reticulocyte count or a parasite count data point; a difference of 108 per 10 counted, or less, is the range of a total red blood cell count data point.

The normal course of infection is shown in figure 1. Of the four animals inoculated with *P. berghei* but not treated with cortisone, all became infected; three recovered spontaneously and one died on the 14th day after infection. No prepatent period was observed. The parasitemia of this group is described on a semi-log scale, the slope of the curve representing the rate of change in parasite numbers. The rate of parasite increase which was rapid during the first 4 days following infection decreased from the 4th to the 13th day after infection. After the 13th day, greater numbers of parasites were destroyed than were produced and the total count decreased. From the 13th to the 17th day after infection, the rate of decrease in parasitemia was less than the rapid decrease, the crisis, which occurred between the 17th and 19th day after infection. After the 19th day, periodic examination for 3 weeks failed to reveal parasites in the peripheral blood. At the end of this time, 0.2 ml of citrated blood from the heart of each surviving rat was inoculated into each of three CF-1 female mice; examination of the mouse blood during the next 30 days failed to reveal parasites. Forty days after infection, 20 mg of cortisone acetate in 4 equal doses over a period of 4 days failed to initiate a relapse in the surviving rats.

A general course of infection can be described in four stages. First there is rapid increase in the number of parasites until the fourth day after infection, followed by a second period in which the increase in parasitemia is less rapid. During the third stage the decline is slow and finally crisis occurs with a rapid decrease in parasite numbers leading to complete disappearance of parasites from the peripheral circulation of the host. This general sequence was also observed in an identical experiment subsequently performed with a group of ten animals.

As shown in figure 1, total red blood cell counts of infected animals not treated with cortisone decrease rapidly until the 13th day after infection due to destruction of parasitized cells. The lowest red cell count coincided with the highest parasitemia. From the 13th to the 25th day after infection, the red cell count rose until it was within normal limits.

The reticulocyte counts of infected animals not treated with cortisone are also shown in figure 1. They remain within the normal range until the 9th day after infection and then rise until, on the 16th day after infection, the number of reticulocytes decreases and the count is within normal limits by the 20th day after infection. The peak reticulocyte count coincided with the beginning of crisis, while the return of reticulocyte counts to normal occurred one day after the disappearance of parasites from the peripheral circulation. Reticulocytes were preferentially parasitized to mature red blood cells.

The course of infection in cortisone treated rats is shown in figure 2. All four animals became infected; three died as a result of infection on the 20th, 21st, and 22nd day after infection. There was no prepatent period. The parasitemia of this group is shown on a semi-log scale. The rate of parasite

increase which was rapid until the 4th day following infection, slowed from the 4th to the 17th day after infection, but was then again rapid until the death of the animals. Near the end of the course of infection, the number of parasites exceeds the number of red blood cells due to pluriparasitism of the cells.

The drop in parasitemia which preceded the recovery of one of the cortisone-treated animals, occurred simultaneously with the terminal rise in parasite numbers immediately prior to the death of the other three cortisone-treated animals. Three weeks after the complete disappearance of parasites from the peripheral circulation of the recovered rat, 0.2 ml citrated blood from this animal's heart was inoculated into each of three female CF-1 mice. Two of the mice displayed no parasites in the red blood cells of their peripheral circulation. The third mouse, after a 5-day prepatent period, developed a parasitemia and died of infection. However, on the 42nd day after infection, 20 mg cortisone acetate in 4 equal doses over period of 4 days failed to initiate a relapse in the rat which served as source of the mouse infection.

As shown in figure 2, the total red blood cell counts of cortisone-treated infected rats, unlike those of the untreated infected animals in figure 1, show an initial rise during the first 2 days following infection. From the 2nd to 18th day after infection, the total number of red blood cells dropped rapidly due to destruction of parasitized cells. The red cell count remained low until death of the three animals which developed fatal infections. Increase in the number of red blood cells on the 18th day after infection, in the one cortisone-treated animal which recovered, coincided with the drop in number of parasites in its peripheral circulation. This animal's total red blood cell count returned to within normal range on the 28th day after inoculation with *P. berghei*.

The reticulocyte counts of infected animals treated with cortisone are also shown in figure 2. They fall below normal on the 3rd day after infection and return to the lower limits of the normal range on the 6th day. Thereafter, the counts alternately remained in the lower limits of this range or fell below normal until the 14th day after infection when the count rose to the higher limits of the normal range. Shortly before the death of these animals, their reticulocyte counts rose above normal and reached as high percentages as those observed in the infected animals not treated with cortisone.

The total red blood cell counts of cortisone-treated noninfected animals are shown in figure 3. These rise above the normal range on the 4th day after the beginning of cortisone injections and remained above normal until the 25th day after the first injection. On a day to day basis reticulocyte counts of these animals were consistently lower, until the 21st day after the first cortisone injection, than those of normal control animals.

DISCUSSION

The studies of Raffaele and Baldi,[3] as well as the work of Galliard and Lapierre[4] on *P. berghei* have demonstrated that there is a mortality of over 50% in young rats, 50 g or less, while in older rats, weighing approximately 120 g, mortality is under 25%.

The young rats in the present experiment, as well as those used in an identical subsequent experiment, weighed approximately 60 g. These experiments

3. Raffaele, G. and Baldi, A. 1950, Rivdi. malariol. **29**: 341–347.
4. Galliard, H. and Lapierre, J. 1951, Bull. Soc. path. exot. **44**: 185–194.

were primarily designed to trace the different courses of *P. berghei* infection in cortisone-treated and untreated rats. Mortality differences also occurred and are recorded. Averaging the results of both experiments, the normal course of *P. berghei* infection in 14 young rats resulted in a mortality of 28% (4 deaths). This figure differs from the 80% mortality (7 deaths) observed in the ten young infected rats treated with cortisone. The infected animals not treated with cortisone which developed fatal infections, died an average of 15.7 days after infection. In cortisone-treated animals, the deaths occurred an average of 20.9 days after infection. An infected rat which received 40 mg cortisone acetate in 4 equal doses over a period of 5 days, i.e., twice the amount of other crtisone-treated animals, survived for 25 days after infection. In the original experiment, the complete disappearance of parasites from the peripheral blood of the one cortisone-treated infected animal which survived occurred 2 days after the disappearance of parasites in surviving rats not treated with cortisone. Such differences between the course of infection and the resultant mortality rate in infected rats treated with cortisone as compared to these in untreated infected animals can be explained by the effect of cortisone on the lymphatic and erythropoietic tissues of the host.[1,5–9]

The common impression that cortisone stimulates erythropoietic activity is based on counts of total red blood cells per given volume of blood. In this experiment, total erythrocyte counts have also been higher for cortisone-treated animals than for untreated control rats. It should be expected that greater erythropoietic activity would primarily increase the number of young red blood cells and only secondarily, if at all, the total number of red blood cells. However, the results of Singer[1] and the present experiment show that cortisone treatment results in a decreased reticulocyte count, while the total erythrocyte count is increased. It must therefore be concluded that cortisone treatment of CF-1 mice and white rats depresses erythropoiesis. The observed increase in total red blood cell counts is probably a result of altered fluid concentrations in the blood stream, rather than of increased cell production.

In the present experiments, the infected white rats treated with cortisone showed a delayed course of infection associated with an increased survival time, but mortality rates higher than those of infected rats not treated with cortisone. The delayed course of infection and increased survival time may be correlated with the lower reticulocyte counts of cortisone-treated animals, resulting from the hormone's depressive effect on erythropoietic tissues.[1] Since reticulocytes are preferentially invaded by *P. berhgei*, it is assumed that they are a more suitable medium for the parasite's growth and reproduction than the more mature red blood cells, or are more readily penetrated. The decreased number of reticulocytes in cortisone-treated animals enhances the host's athreptic immunity by depriving the parasite of its optimal substrate during the initial course of the infection. The final rise of parasite counts in cortisone-treated animals

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which began on the 17th day after infection, may be attributed to the rise of reticylocyte counts above normal range which had begun on the previous day in these animals. Reticulocyte counts in uninfected animals treated with cortisone also rise at about this time so that we may conclude that the depressive action of cortisone on erythropoiesis begins to wane, permitting increased production of reticulocytes in infected animals whose red cells have been depleted.

This rise in reticulocyte counts and the subsequent rise in parasite counts does not completely explain the higher incidence of mortality in cortisone-treated infected animals. Despite the excellent conditions for parasite growth and reproduction offered by large numbers of reticulocytes, infected animals not treated with cortisone were able to overcome the infection by reducing the number of parasites in their blood streams.

In an attempt to explain the higher mortality of infected animals treated with cortisone, we may consider the hormone's "inhibition of proliferative activity" of lymphopoiesis.[1] The lymphatic tissue are the source of both the cellular (lymphocytes and hence the phagocytic macrophages which ingest the parasite) and the possible humoral defense mechanisms of acquired immunity against *Plasmodium*. These tissues are involuted by cortisone,[1] thus leaving the host, in the majority of cases, defenseless against the parasite.

Taliaferro and Taliaferro,[10] in their work on *P. brasilianum* reported the occurrence of abnormal, so-called crisis forms of plasmodia. In the case of *P. berghei* in the white rat, abnormal forms are seen at the time of and following crisis. These crisis forms have nuclei similar to those of normal *P. berghei* parasites, but possess a poorly staining cytoplasm which is difficult to distinguish within the red cell.

SUMMARY

The effect of cortisone on the course of malarial infection with *Plasmodium berghei* in the white rat is twofold.

Cortisone initiates a delayed course of infection and an increased survival time for the host. This effect is ascribed to an enhancement of the host's athreptic immunity by depressing erythropoiesis and thus decreasing the rate of formation of reticulocytes which are the parasite's optimal substrate.

Also, in infections treated with cortisone mortalities are higher than in infections not treated with cortisone. This result has been ascribed to cortisone's involution of the host's lymphatic tissues, source of the cellular and possible humoral defense mechanism of acquired immunity to the parasite.

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EXPERIMENTAL HISTOPLASMOSIS IN MICE, AND PREGNANCY

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Reports of transplacental transmission of infection with *Histoplasma capsulatum* are lacking. The present report is concerned with efforts made to determine whether or not this phenomenon may be successfully accomplished experimentally in mice.

MATERIAL AND METHODS

Two groups, each of 5 adult white female mice weighing 20 g, were used in the experiment. Group I, consisting of 5 pregnant animals, were inoculated intravenously with a heavy suspension of the mycelial phase of *H. capsulatum* 5 to 12 days before termination of pregnancy. On the date of delivery the pregnant animals were sacrificed and cultures were prepared from the maternal liver and spleen and from the placenta. Cultures were also taken from the livers of the fetuses. All cultures were grown on Sabouraud's glucose agar at 20 C for 8 weeks.

Group II consisted of 5 animals which were inoculated with a heavy suspension of the mycelial phase of *H. capsulatum*. After an interval of 2 to 3 weeks these animals were mated. Five animals survived the disease long enough to be killed on the expected date of delivery. Cultures were prepared as in group I. In addition, the uterus was also cultured.

The pregnant animals in groups I and II were sacrificed close to term. The mice were pinned down on a parafin tray. They were thoroughly soaked with 95% ethyl alcohol. The animal was then opened with scissors and the skin reflected in such a way that fragments of liver were easily removed for culture.

The external surface of the uterus was sterilized with 95% alcohol. With sterile instruments the uterus was opened and a piece of placenta was taken and cultured on Sabouraud's medium. The fetus was opened and a piece of fetal liver was also cultured. Another set of sterile instruments was used to open the fetus and another set of instruments to make the incision of the fetus and for the removal of liver.

The only difference in technique between groups I and II was the fact that the placentas and the fetuses were cultured in group I in situ, whereas in group II the uterus was removed in toto before it was incised.

Group III animals were inoculated with a heavy suspension of the mycelial phase of *H. capsulatum*. Fifteen nonpregnant animals were included in this study. They subsequently became pregnant and delivered young from 3 weeks to 5 months after they had been injected. When the litters were approximately 3 weeks old, the family was killed and cultures were prepared from the liver and spleen of both the mother and offspring. The same method of taking the cultures from the mother and offspring was used in this group as in groups I and II. None of the tissue was triturated before it was placed on Sabouraud's medium.

RESULTS

In groups I and II four positive cultures were obtained from fetal liver but the overwhelming majority of fetal organs gave negative results (see table 1).

Group III: The results of cultures of female mice injected 3 weeks to 5

TABLE 1.—*Results of cultures from maternal and fetal organs in experimental histoplasmosis.*

		Maternal liver and spleen	Placenta	Uterus	Fetal liver
Group I—animals injected during pregnancy	1.	+	+		3 −
	2.	+	+		2 + & 1 −
	3.	+	−		8 −
	4.	+	−		3 −
	5.	+	3+		3 −
Group II—animals injected prior to pregnancy	6.	+	+	−	1 + & 4 −
	7.	+	4+	3+	7 −
	8.	+	3+	2+	5 −
	9.	+	2+	2+	1 + & 5 −
	10.	+	4+	3+	4 −

+ Denotes positive culture, if more than 1 was obtained the number is indicated.
− negative culture.

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months prior to pregnancy and killed 3 weeks after delivery were the following. All maternal livers and/or spleens yielded positive cultures. None of the cultures of the liver or spleen from the 86 offspring yielded *H. capsulatum*. (A minimum of two and a maximum of nine young were cultured per family.)

DISCUSSION

It is noted that in group I all animals were pregnant before *H. capuslatum* was injected and the animals in group II were all inoculated with the organism before they became pregnant. All the maternal animals in group I gave positive cultures from the liver and spleen. Fewer positive cultures were obtained from the placentas of group I than from group II.

Of 47 cultures obtained from fetal livers, 4 gave positive results. This is contrary to all the other data in the experiment. The technical difficulties in opening the uterus in a sterile manner in order to deliver the placenta and the fetuses were such that we believe contamination was the likely source of the four positive cultures. To prove this point cultures were taken from the uterine wall and were almost uniformly positive. Since it was impossible to be certain that the surface sterilization of placenta and fetus were adequate even after dipping in alcohol, it is felt that the positive cultures obtained from 4 fetal livers probably represent contaminations carried over from the uterus or maternal blood with our instruments.

We came to this conclusion in view of the negativity of so many livers in group III in which cultures could be taken under much better conditions. This was further demonstrated by the absence of organisms upon microscopic examination of the livers. *H. capsulatum* was seen in the placentas in groups I and II in which positive cultures were obtained. In group III all the mothers had positive liver and/or spleen cultures for *H. capsulatum* and the organism was identified histologically in the liver and spleen. None of the offspring yielded a positive culture from either the liver or spleen and microscopic examination of the organs revealed no organisms.

Since we had the impression that infected animals become pregnant less frequently than normal mice, a supplementary experiment was started with a group of 24 female mice which were exposed to males which were exchanged every 4 to 5 days. In this manner the same male animals were used for the same group of infected and uninfected mice. No vaginal smears were made in either group and the same female mice were exposed 2 to 3 weeks to males. Seven of the uninfected female mice became pregnant with a total of 44 young; four of the infected group became pregnant with 25 young. We do not not consider this conclusive evidence that infection with *H. capsulatum* influences the fertility of mice.

SUMMARY

In a group of 10 pregnant animals infected with *Histoplasma capsulatum*, 19 placentas from 8 mice showed the organism present in the placenta. Positive liver cultures from 4 fetuses are believed to be the result of contamination during handling of the minute organs.

H. capsulatum was not demonstrated in 86 young mice killed 3 weeks after delivery. They were offspring of 15 animals injected intravenously with *H. capsulatum* 3 weeks to 5 months prior to pregnancy.

VIRULENCE OF *BACTERIUM TULARENSE*

I. A STUDY OF THE VIRULENCE OF *BACTERIUM TULARENSE* IN MICE, GUINEA PIGS, AND RABBITS

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Differences in virulence of strains of *Bacterium tularense* for various species of animals have been recognized.[1–3] Davis, Philip, and Parker[1] noted that guinea pigs did not furnish an accurate measure of the virulence of a recent isolate because these animals are highly susceptible to infection. They suggested the use of domestic rabbits, which are more resistant.

Francis and Felton[4] have proposed a measure of virulence, based on the susceptibility of mice, guniea pigs, and rabbits to infection with this organism, which appears to be the most direct and valid of any that have been applied. Approximately 1 to 10 organisms of maximum virulence are sufficient to cause fatal infection in mice, guinea pigs, and rabbits. Diminished virulence first shows itself in the failure of the organisms to kill rabbits. This method of assessing virulence has the marked advantage that the number of organisms necessary to kill each species of animal may be given and thus a quantitative designation of virulence for the various experimental animals may be assigned to a strain.

It has been observed by Philip and Davis[2] that the period of survival of guinea pigs infected with *Bact. tularense* varies with the strain employed. The number of organisms contained in the inoculum was thought to have no material influence on the length of this period. Green[5] also reported that the period of survival of guinea pigs distinguishes the virulence of strains of this organism. He stated that several isolates of *Bact. tularense* from snowshoe hares actually were grouse strains because guinea pigs infected with them survived for long periods. However, in the course of studies at the Rocky Mountain Laboratory,[6] the observation was made that the number of organisms injected into mice by the subcutaneous route has a marked effect upon the survival time of these animals.

In an effort to standardize a measure of virulence, several isolates of *Bact. tularense* have been studied in mice, guinea pigs, and rabbits by applying the method of Francis and Felton on a quantitative basis and by determining the period of survival of animals infected with the various strains tested. The results obtained are presented in this report.

MATERIALS AND METHODS

Eight isolates of *Bact. tularense* were chosen: (1) 425 F_4G is a strain originally isolated from an engorged female *Dermacentor andersoni* in May 1941, standardized by lyophilization in December 1952, and employed in this laboratory for testing

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potency of tularemia vaccines; (2) 425 $F_{15}G$ was derived from the former by serial passage through 11 guinea pigs; (3) 425 $F_{30}G$ was derived from 425 $F_{15}G$ and was passed serially through 15 guinea pigs; (4) 425 RV_{50} was derived from 425 F_4G and has been subcultured serially on glucose-cystine-blood agar for 50 transfers; (5) 425 M_2T_2 was also derived from 425 F_4G, but had exhibited marked increase in virulence after alternate transfers through mice and ticks; (6) Nevada 14 was isolated from a hare (*Lepus californicus*) and had undergone varied experiences in the laboratory; (7) AC is presumed to be derived from Nevada 14, as it was isolated from a patient who had worked with ticks infected with that organism just prior to his illness; and (8) Schu SD was obtained from Eigelsbach and had been isolated from a finger ulcer by Foshay in 1941. With the exception of 425 F_4G, each of the above isolates was tested once. Duplicate tests were performed with 425 F_4G. In addition, the intraperitoneal and subcutaneous routes of infection were compared. Isolates Schu and 425 $F_{30}G$ were used in the determination of the period of survival of infected guinea pigs.

Female mice about 21 days old from stock maintained at the Rocky Mountain Laboratory were used. Guinea pigs and rabbits were obtained from local breeders. The guinea pigs weighed about 1 pound and the rabbits about 3 pounds.

The bacterial suspensions were prepared by rehydration of lyophilized material, which was then cultured on glucose-cystine-blood agar. The resultant growth was transferred to slants of the same medium and incubated for 24 hours at 37 C. The bacteria were harvested in saline and washed once. The mass obtained after the second centrifugation was suspended in saline and shaken with glass beads to prepare the stock suspension. The standard suspension (10^9) was prepared from stock by the method of Francis.[7]

Serial 10-fold dilutions, ranging from 10^1 to 10^9, were immediately made in saline. Three glucose-cystine-blood agar plates were inoculated with 0.1 ml each of the four dilutions that, through previous experience, were expected to yield countable plates. Density determinations were also done on the 10^1 suspensions with the use of a Klett photometer with no filters.

Ten rabbits, 10 guinea pigs, and 36 mice were inoculated subcutaneously with measured amounts of material from each of the dilutions from 10^0 to 10^9. For mice, the inoculum consisted of 0.03 ml administered at the base of the tail; for guinea pigs, 0.1 ml given in the nuchal area; and for rabbits, 0.1 ml inoculated subcutaneously in the ear with consequent formation of a bleb. In one experiment (exp. 10) all animals were inoculated by the intraperitoneal route. The animals were observed at approximately the same time once a day for 14 days or more, and the average days of survival calculated.

7. Francis, E. E. and Evans, A. C. 1926, Pub. Health Rep. **41**: 1273.

RESULTS

Ten experiments were performed with eight isolates of *Bact. tularense* in order to determine the relative virulence of each for mice, guinea pigs, and rabbits. If the number of bacteria at 10^0 dilution is divided by the number of LD_{50}'s at the same dilution (table 1), it will be seen that the number of bacteria in an LD_{50} for mice ranges from 0.5 to 3.7 organisms. Data on the virulence of *Bact. tularense* derived from determinations of the LD_{50} on mice alone would not serve to differentiate between the isolates.

Since it may be confusing to depict the LD_{50} for each species of animal in terms of actual numbers, the characteristic of the logarithm of the number of bacteria that produces lethal infection has been selected as the most convenient term with which to express the LD_{50}. Thus, the virulence of Schu SD can be stated as M = 0, G = 0, R = 0, indicating that 1 to 9 organisms are capable of producing lethal disease in mice, guinea pigs, and rabbits. For 425 $F_{30}G$, the expression M = 0, G = 1, R = 8 states that 1 to 9 organisms will kill mice, 10 to 99 will kill guinea pigs, and 100,000,000 to 999,999,999 organisms are required to kill rabbits. It is evident that the various isolates can be divided into three groups as regards their relative virulence for the three hosts studied. Nevada 14, Schu SD, 425 M_2T_2, and AC are all fully virulent for each of the three species of experimental animals. It is apparent, however, that 425 $F_{30}G$ possesses considerable virulence for guinea pigs, in contrast to 425 F_4G, 425 RV_{50}, and 425

TABLE 1.—*A comparison of the virulence of several strains of* Bact. tularense *as determined by the method of Francis and Felton.*

Experiment	Strain	Bacterial count (0.1 ml)*	LD_{50} mouse (calculated for 0.1 ml)*	Number of organisms constituting one LD_{50}		
				Mouse	Guinea pig	Rabbit
1	Schu SD	4.6×10^{8}	2.7×10^{6}	10^{8}	10^{9}	10^{9}
2	Nev. 14	5.5×10^{8}	9.7×10^{8}	10^{8}	10^{9}	10^{9}
3	AC	5.0×10^{8}	3.2×10^{8}	10^{8}	10^{8}	10^{9}
4	$425M_3T_2$	5.6×10^{8}	5.3×10^{6}	10^{8}	10^{8}	10^{9}
5	$425F_{15}G$	4.7×10^{8}	7.6×10^{6}	10^{8}	10^{8}	10^{8}
6	$425F_{15}G$	1.2×10^{9}	1.7×10^{8}	10^{8}	10^{8}	$>10^{10}$
7	$425F_4G$	3.0×10^{8}	1.9×10^{8}	10^{8}	10^{8}	10^{9}
8	$425F_4G$	4.1×10^{8}	1.1×10^{8}	10^{8}		10^{9}
9	$425RV_{50}$	3.0×10^{8}	1.1×10^{8}	10^{8}	10^{7}	$>10^{9}$
10 IP	$425F_4G$	5.3×10^{8}	9.7×10^{8}	10^{8}	10^{8}	10^{8}

* Of 10^{8} dilution.

$F_{15}G$, which require at least 10,000 organisms to constitute an LD_{50} for these animals.

The period of survival of mice inoculated with the various isolates of *Bact. tularense* was calculated (table 2). When large numbers of organisms are employed to infect mice by the subcutaneous route, it is not possible to differentiate between isolates of different degrees of virulence. The survival time of mice infected with approximately 10^{8} organisms of isolates 425 F_4G and Schu SD is 2.6 days in each instance, although the results of the previous method of testing for virulence demonstrated a considerable difference between the two isolates. A marked difference became apparent when the number of organisms used to produce infection was decreased a hundredfold. The period of survival increased to 6.0 days for mice given inoculums of 425 F_4G and 3.4 days for those given Schu SD. It is possible to differentiate the isolates into groups rather easily when the number of organisms in the inoculum is about 100 to 1000. Isolates Schu SD, AC, Nevada 14, and 425 M_3T_2 have survival periods of less than 6 days, those receiving isolates 425 F_4G and 425 RV_{50} survived for periods of about 8 days, and mice inoculated with isolates 425 $F_{15}G$ and 425 $F_{30}G$ have survived for periods of intermediate length. Figures 1 and 2 depict the survival period among mice infected with graded amounts of three isolates of *Bact. tularense.* It is possible to differentiate these isolates on the basis of survival period of mice, especially if small numbers of organisms are employed to infect the mice.

Similar results are obtained following subcutaneous injection of graded doses of organisms into guinea pigs (table 3). The survival period of guinea pigs infected with Schu SD is much less than that for animals infected with 425 $F_{30}G$.

TABLE 2.—*The period of survival of mice inoculated with various strains of* Bact. tularense *in relation to the size of dose.*

Exp.	Strain	Inoculum (0.03 ml) Number of organisms in 10^{6} dilution	Inoculum (0.03 ml) Number of LD_{50}'s in 10^{6} dilution	Days of survival of mice given graded inoculums							
				10^{6}	10^{5}	10^{4}	10^{3}	10^{2}	10^{1}	10^{0}	10^{-1}
1	Schu SD	14×10^{7}	8.1×10^{7}	2.6	3.1	3.4	4.1	4.3	4.6	5.4	6.0
2	Nev. 14	16×10^{7}	29×10^{7}	3.0	3.4	3.9	4.2	4.7	5.0	5.5	6.2
3	AC	15×10^{7}	9.6×10^{7}	2.8	3.3	4.0	4.1	4.8	5.3	5.7	
4	$425M_3T_2$	17×10^{7}	16×10^{7}	2.5	3.2	4.0	4.2	4.6	4.7	5.5	6.1
5	$425F_{15}G$	14×10^{7}	23×10^{7}	2.9	3.9	4.3	4.8	6.2	6.2	6.9	8.1
6	$425F_{15}G$	36×10^{7}	52×10^{7}	2.6	3.2	4.4	4.8	5.2	5.6	6.2	7.0
7	$425F_4G$	10×10^{7}	6×10^{7}	3.2	4.0	4.8	5.7	6.3	7.2	7.9	8.9
8	$425F_4G$	12×10^{7}	3.3×10^{7}	2.6	3.7	6.0	5.8	6.5	8.1	8.2	9.3
9	$425RV_{50}$	10×10^{7}	3.6×10^{7}	3.0	3.9	4.5	5.6	6.1	7.2	8.8	
10	$425F_4G$ IP	16×10^{7}	29×10^{7}	2.0	2.1	3.0	3.1	3.9	4.0	4.1	4.9

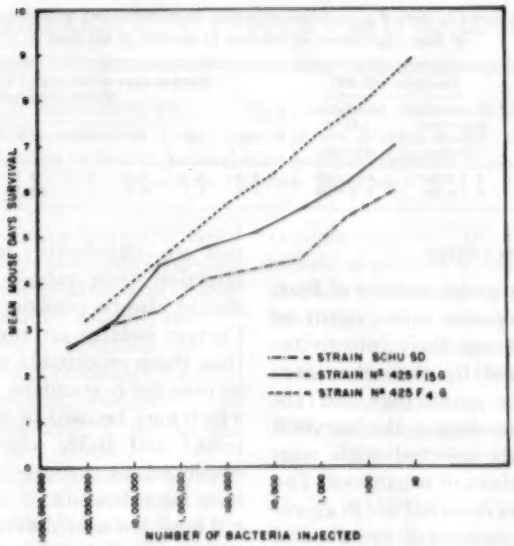

FIG. 1.—Virulence and dosage (bacterial count) in relation to survival period of mice.

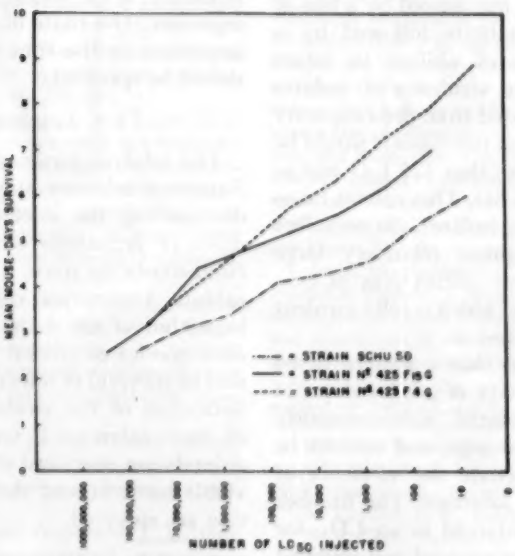

FIG. 2.—Virulence and dosage (LD_{50}) in relation to survival period of mice.

TABLE 3.—*The period of survival of guinea pigs inoculated with two strains of* Bact. tularense *in relation to the size of the dose.*

Exp.	Strain	Inoculum (0.1 ml)		Average days of survival of guinea pigs given graded inoculums							
		Number of organisms in 10^6 dilution	Number of LD_{50}'s in 10^6 dilution	10^0	10^1	10^2	10^3	10^4	10^5	10^6	10^7
1	Schu	6.5×10^8	4×10^6	3.4	4.2	4.8	5.7	5.9	6.3	6.5	7.6
2	$425F_4G$	4.7×10^8	5.0×10^7	5.0	5.7	8.5	7.7	10.7	13.6	12.9	12.8

DISCUSSION

It is possible to assign isolates of *Bact. tularense* to categories with regard to virulence by testing their infectivity, when administered by the subcutaneous route, for mice, guinea pigs, and rabbits and by determining the survival period of animals infected with relatively small numbers of organisms. The results of the tests reported are in agreement with the statement of Francis and Felton concerning the virulence of *Bact. tularense*. Fully virulent organisms may be defined as those which produce fatal infections in mice, guinea pigs, and rabbits when 1 to 9 organisms are given by the subcutaneous route. Decreasing virulence is first manifested by a loss of infectivity for rabbits followed by a progressive loss of ability to infect guinea pigs. The virulence of isolates cannot be estimated from their capacity to kill mice, for on this basis it would be necessary to agree that 425 F_4G was as virulent as Schu SD. This cannot be so since the former isolate fails to infect rabbits and requires relatively large numbers to infect guinea pigs in contrast to the latter, which is fully virulent for the animals tested.

It is suggested that a system based upon the infectivity of graded doses of organisms inoculated subcutaneously into mice, guinea pigs, and rabbits be adopted to designate the virulence of isolates of *Bact. tularense*. The number of organisms contained in an LD_{50} for each species is ascertained and rounded to the nearest whole logarithm. Since rats are considerably more resistant to infection than rabbits, these animals should also be considered in such tests. Certain isolates are much less virulent than those reported in this paper. These include such standard isolates as Jap, which may be used in mouse-protection tests,[8] and B-38, which is ordinarily employed as antigen in agglutination tests for tularemia.

There are also differences in the period of survival of animals infected with isolates of *Bact. tularense* of different degrees of virulence. It would, however, be difficult to place a value on the length of the survival period that would be helpful in defining the precise degree of virulence of a single isolate of this organism. The route of infection is also important to the time of survival and should be specified.

SUMMARY

The relative virulence of isolates of *Bacterium tularense* may be assessed by determining the infectivity of graded doses of organisms administered subcutaneously to mice, guinea pigs, and rabbits. A numerical value based on the logarithm of the LD_{50} is assigned for each species of animal tested. The period of survival of infected animals is an indication of the virulence of a strain of *Bact. tularense* if large numbers of animals are used and if the number of viable bacteria and the route of infection are specified.

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VIRULENCE OF *BACTERIUM TULARENSE*

II. EVALUATION OF CRITERIA OF VIRULENCE OF *BACTERIUM TULARENSE*

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Criteria which have been employed in efforts to determine the relative virulence of different strains of *Bacterium tularense* include animal tests,[1–5] colonial morphology,[4] electrokinetic reactions,[3,4] antibiotic resistance,[6] mutation and population effects,[4,7] and immunogenic potency.[8–10] An earlier communication from this Laboratory[1] described a modification of the technic of Francis and Felton[2] which has been found to be useful. The present report is concerned with an extensive comparison of these various methods for estimating the virulence of *Bact. tularense* and an assessment of their validity.

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MATERIALS AND METHODS

The eight strains of *Bact. tularense* studied in the work described in the previous communication[1] were likewise used in the present studies (strains Nevada 14, AC, Schu, 425-F_4G and its derivatives, F_{15}G, F_{30}G, RV_{50}, and M_8T_2). In addition, ten other strains or substrains were employed,—425-RV_{100}, derived from 425-RV_{50} by 50 serial subcultures on cystine glucose blood agar (CGBA); 38, a strain which had become completely avirulent in the laboratory, and 38-NS derived from it by the method of Eigelsbach, Braun, and Herring;[4] 41804A-Rough, a rough avirulent strain from the laboratory stock collection; Jap, isolated in 1926 from an ulcer of a human case of tularemia and since become avirulent, and three smooth variants and one "non-smooth" variant derived from it; and Norway, a strain of moderate virulence isolated in fall of 1953 from a tick, *Ixodes ricinus*, removed from a sick hare in Norway and sent to this Laboratory.

Colonial morphology was determined by the method of Eigelsbach, Braun, and Herring,[4] i.e., inoculation of cystine glucose agar (CGA) plates and observation of the colonies by transmitted oblique light under a dissecting microscope.

Saline agglutination was performed by the method of Avi-Dor and Yaniv[6] and acriflavine

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agglutination according to Eigelsbach, Braun, and Herring.[4]

Growth rates of the various strains were compared by simultaneous inoculation of CGBA plates and simultaneous photography of the resulting colonies to indicate volumes of bacteria. Numbers of bacteria in these colonies were determined by suspending them in a standard amount of saline per colony and making plate counts of the resulting suspensions.

Inhibition of the growth of virulent by avirulent organisms was tested by four methods:

1. The block-inoculation plate method of Yaniv and Avi-Dor:[3] Plates were seeded by placing small strips of filter paper (Whatman no. 3), saturated in a standardized suspension of the strain to be tested for ability to inhibit, in pairs opposite each other with a space equal to the width of the strip between them. These were left on the plate for 5 minutes, removed, and the plates incubated. After 24 or 48 hours incubation, a filter-paper strip, saturated in a standardized suspension of the strain to be tested for susceptibility to inhibition, was laid across the gap between the previously incubated blocks, left 5 minutes, removed, and the plates were reincubated. After 24 hours, the plates were examined for inhibition of growth of the center strips by the side blocks independently by three observers, two of whom did not know what strains were involved in the experiment.

2. Strain 425-F_4G was subcultured on CGBA at intervals of 2 to 3 days. If less virulent organisms developed, it was considered that they might have an increased growth rate and thus decrease the virulence of the original culture.[4]

3. Strains Nevada 14, Schu, 425-F_4G, and 425-M_3T_2 were subcultured every other day for 32 consecutive passages on a medium consisting of 60% egg-yolk and 40% saline, solidified by heating to 80 C for 1 hour. In addition, a culture of the Schu strain which had previously undergone 32 such subcultures was passed simultaneously. Following this, a virulence titration in animals was performed on each 32nd subculture.

4. A standardized suspension of the fully virulent Schu strain was mixed in equal proportions with similar suspensions of each of two avirulent strains, smooth 38 and rough 41804A. The resulting mixtures were subcultured daily on CGBA for 10 transfers. The 10th subcultures were then tested for virulence by the method previously described. A similar 10th subculture of Schu alone served as control.

Antibiotic sensitivity of the Schu, Nevada 14, 425-F_4G, 41804A-Rough, and 38 strains was tested by spreading 0.3 ml of a standard dilution of suspensions over the surface of CGBA plates. These were allowed to dry at room temperature for 2 hours. A "Desi-Disc" (National Bio-Text Corporation) containing 10 μg of streptomycin or 10 μg of aureomycin was placed in the center of each plate. After 3 days incubation at 37 C, the rings of inhibition were measured.

The relative amounts of protective antigen in strains Schu, Nevada 14, 425-F_4G and 38 of *Bact. tularense* were assayed by the specific antigen precipitation method and the mouse protection test. For this purpose, the organisms were cultured in Blake bottles on CGBA. The organisms from one 24-hour slant were used as the inoculum for each Blake bottle except in the case of strain 38 where 48-hour inoculum was used. After 48 hours incubation at 37 C, the organisms were flushed off the agar surface and washed four times by centrifugation in 0.15 *M* sodium chloride. The resulting suspension was precipitated by an excess of cold acetone, allowed to stand 1 hour at 6 C, and the acetone removed by filtration through a Buchner funnel. The precipitate was then washed with more cold acetone. Residual acetone was removed by evaporation at room temperature. The precipitate was placed in a vacuum desiccator over calcium chloride at room temperature for 24 hours, and then weighed. One percent suspensions were made in saline.

Three consecutive extractions of the 1% suspensions were made as follows: To 50 ml of the 1% suspension, 2.5 volumes of ethyl ether were added, the mixture vigorously shaken and allowed to stand at room temperature for 24 hours with occasional shaking. The aqueous phase was collected and centrifuged at 4500 r.p.m. for 1 hour in an International #2 angle-head centrifuge. The supernate was removed and made up to 50 ml with 0.15 *M* sodium chloride, the sediment was likewise restored to volume and again extracted with ether. After two further extractions, the residue was taken up in a small volume of 0.15 *M* sodium chloride and treated in the Mickle apparatus for 30 minutes to disintegrate the particulate remains of the organisms. The resultant mixture was centifuged as before, giving a supernate and a sediment, each of which was made to 50 ml. Thus, four supernatant fluids and one resuspended residue were obtained for each strain of *Bact. tularense* studied.

The antigen content of the supernates was estimated by capillary-tube precipitation titration,[11] employing a standard rabbit antiserum pre-

11. Ormsbee, R. A., Bell, J. F. and Larson, C. L. 1955, Studies on *Bacterium tularense* antigens. I. The isolation, purification, and biologic activity of antigen preparations from *Bacterium tularense*. I. Immunol. **74**: 351–358.

pared against strain Nevada 14 antigen. The sediments which remained after disintegration in the Mickle apparatus followed by centrifugation were resuspended and titrated in the same manner. Since it has been shown that the degree of protection afforded mice immunized with antigens prepared by the ether extraction technic was proportional to the antigen precipitation titer, the supernates and resuspended sediments were then tested by the mouse protection test to determine their protective antigen content.[12] This test was performed by injecting 21-day-old mice with a fixed dose of antigen. After 5 days, the mice, in groups of six, were challenged with serial decimal dilutions of a standardized suspension of strain 425-F_4G and the LD_{50} compared with that of nonimmunized controls. The difference in LD_{50} values between the immunized and nonimmunized mice was taken as a measure of the protection afforded by the antigen.

RESULTS

Colonial morphology.—Of the strains tested, only frankly rough 41804A, the "nonsmooth" 38 NS_1, and Jap S_4NS did not yield the smooth type of colonies. Strain 425-RV_{50} produced occasional colonies with much folded surfaces, but these were very infrequent and reverted immediately and apparently completely to the parent type on subculture. All others regardless of degree of virulence yielded the smooth type of colony. However, it was noted that, while the fully virulent (Nevada 14, AC, Schu, and 425-M_3T_2) and the moderately virulent (Norway) strains yielded colonies which were of uniform size for each strain, there was considerable variation in those of the Jap, the 38, and the other 425 strains. This difference was also apparent on CGBA plates and is illustrated by comparison in figure 1 of strains Schu and Nevada 14 with 38 and 425-RV_{100}. It is apparent that the virulence of a new culture cannot be accurately assessed by inspection of colonies.

Cell surface electrokinetic phenomena.—Of the 18 strains tested, only the

12. Bell, J. F., Larson, C. L., Wicht, W. C. and Ritter, S. S. 1952, Studies on the immunization of white mice against infections with *Bacterium tularense*. J. Immunol. **69**: 515–524.

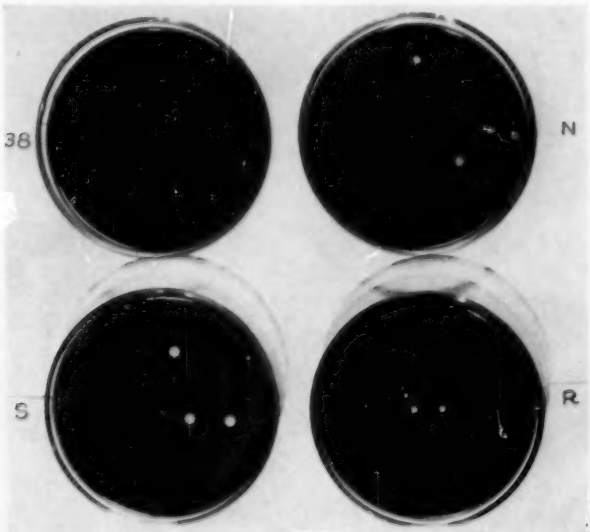

FIG. 1.—Colony size of the 38 (upper left), Nevada 14 (upper right), Schu (lower left), and 425-RV_{100} (lower right) strains of *Bact. tularense*.

TABLE 1.—*Maintenance of level of virulence of* Bact. tularense *425 on CGBA.*

Substrain of strain 425	Log (number organisms necessary to kill) (LD_{50})		
	Mice	Guinea pigs	Rabbits
F_4G	0	5	9
RV_{50}	0	7	9
RV_{100}	0	4	9

TABLE 2.—*Reduction of virulence of four strains of* Bact. tularense *on egg-yolk medium.*

Strain	Number of subcultures	CGBA count	Log (number of organisms necessary to kill) (LD_{50})		
			Mice	Guinea pigs	Rabbits
Nevada 14	0	5.5×10^8	0	0	0
	32	9.5×10^7	1	>9	>9
425-F_4G	0	3.0×10^8	0	5	>9
	32	7.3×10^7	1	8	>9
425-M_1T_1	0	5.6×10^8	0	0	0
	32	2.2×10^8	0	<3	>9
Schu	0	4.6×10^8	0	0	0
	32	4.6×10^8	2	<3	9
	64	7.0×10^8	>4	>7	>7

frankly rough 41804A strain was agglutinated by acriflavine. With the *N*/160 saline solution of Avi-Dor and Yaniv,[3] the 41804A and Jap S3 strains were agglutinated; however, the water controls were also positive and with the Jap S3 strain this proved to be due to lack of electrolyte, as the organisms became more and more clumped with each washing in distilled water. Thus, cell surface phenomena are not reliable criteria of the virulence of *Bact. tularense.*

Loss of virulence on artifical mediums.—Table 1 illustrates the failure of 50 and of 100 consecutive subcultures on CGBA to cause any marked decrease of virulence in strain 425-F_4G. These findings were reported in greater detail previously.[1] A slight (2 log) difference in the LD_{50} for guinea pigs is apparent after 50 subcultures, but the virulence after 50 subsequent subcultures was the same as that of the parent strain.

Table 2 illustrates the rather marked loss of virulence brought about in four strains of *Bact. tularense* by serial subculture on coagulated egg-yolk-saline medium, as compared with the virulence of the same strains before such passages. This reduction of virulence is particularly marked in the highly virulent Nevada 14 and Schu strains, but the difference from the parent strains is apparent in all cultures.

It was also noted that the colonies of the Nevada 14 strain after 32 subcultures and of the Schu strain after 64 were much smaller and more irregular in size than those of the parent strains. The colonial morphology of the three other terminal subcultures was typical of the parent strains. Thus, there was

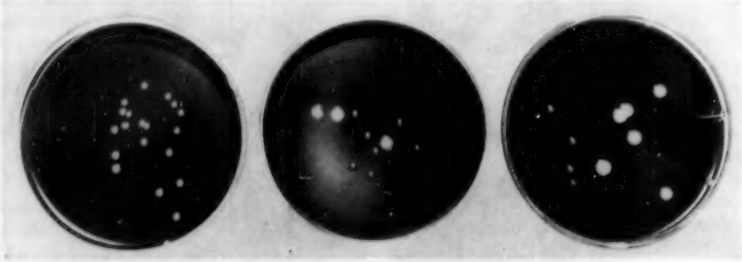

FIG. 2.—Mixture of standard suspensions of *Bact. tularense* Schu (virulent, large white colonies) and 41804A-rough (avirulent, smaller dark colonies) on CGBA. Original mixture (left) after one subculture (center) and after 10 subcultures (right). The tubes containing the original mixture and the first subculture were refrigerated during 10 days while the further subcultures were accomplished; all mixtures were then subcultured once and the plates inoculated simultaneously.

little correlation between loss of virulence and the change of colony type.

Rate of growth.—It has been suggested by Eigelsbach, Braun, and Herring[4] and by Yaniv and Avi-Dor[7] that diminution of virulence of cultured bacteria occurs through overgrowth of the virulent by the avirulent variants in the strain. In our experience this would not be constant. In fact, the reverse may be true with some cultures, as illustrated in figure 1, in which it is apparent that the virulent Schu and Nevada 14 strains produce larger colonies than the moderately virulent 425-RV_{100} and much larger colonies than the avirulent 38 strain when inoculated simultaneously onto CGBA.

Table 3 illustrates the fact that this difference in colony size is due to difference in numbers of bacteria in the colonies and thus to rate of growth. When the colonies on plates, such as those shown in figure 1, are suspended in a standard amount of saline per colony and counts are made, the average numbers of organisms per colony decrease with the size of the colonies, culminating in a greater than 2-log difference between strains Schu with the largest colonies and 38 with the smallest.

Overgrowth of virulent by avirulent strains.—It has been suggested (Yaniv and Avi-Dor;[7] Eigelsbach, Braun, and Herring[4]) that avirulent bacteria inhibit and overgrow virulent by production of a substance which is antagonistic to the virulent organisms, and this was demonstrated by Yaniv and Avi-Dor. However, in our experience, any strain (except 38) seeded between preincubated blocks of the same or any other strain was inhibited and failed to grow between the blocks. When 38 was thus tested, growth was neither inhibited nor enhanced.

TABLE 3.—*Average numbers of organisms in colonies of different strains of* Bact. tularense.

Strain	Average number organisms per colony
Schu	5.4×10^9
Nevada 14	3.3×10^9
425-RV_{100}	1.4×10^9
38	2.6×10^7

Mixtures of approximately equal numbers of virulent (Schu) and avirulent (38 and 41804A-Rough) strains were made in saline, inoculated onto CGBA slants, and subcultured 10 times on this medium. Aliquots of the original mixture and of the 1st and 10th subcultures were cultivated on CGBA. The results, as illustrated in figure 2, indicate that the avirulent strains failed to suppress the virulent. Strain 41804A, which has a more rapid growth rate than strain 38 also had a greater survival rate, but was, nonetheless, unsuccessful in competing with the virulent Schu strain.

As further evidence of the failure of avirulent to inhibit virulent strains, virulence tests in animals[1] were performed on the 10th subcultures of the above mixtures and compared with those of a 10th subculture of strain Schu alone. CGBA plates, inoculated with appropriate dilutions of the suspensions used for test, yielded colonies of different morphology for the strains used in each mixture (see fig. 2) and thus the approximate proportions of the organisms could be determined. Table 4 summarizes the findings of these

TABLE 4.—*CGBA counts and mouse LD_{50}'s of 10th subcultures of mixtures of virulent (Schu) and avirulent (38 and 41804-A-rough) strains of* Bact. tularense.

Mixed culture	Original colony count 10^7 Schu/other	10th subculture colony Schu/other	Mouse LD_{50}	Number of bacteria in 1 LD_{50}
Schu/38	21/4	44/0.7	8.8	1–10
Schu/41804-A-rough	25/12	8/3	7.8	1–10
Schu		43	8.7	1–10

experiments. It will be noted that the proportions of the avirulent strains, especially 38, in the original mixtures tend to be low when compared with that of Schu. This is due to the fact that strain 38 appears to be of low viability, so that standard suspensions arrived at by optical density always yield lower counts than do other strains at the same density. With the rough strain, 41804A, autoagglutination causes variation in counts of the standards. It can be seen from the table that the virulent Schu strain completely dominated the avirulent strains in numbers and in the effect in the animals, in contrast to the findings of previous authors. It would therefore be impossible to assess accurately the virulence of a culture by its rate of growth as compared with other strains or by its inhibition of or by other strains.

Antibiotic sensitivity.—Table 5 lists the sizes of the zones of inhibition obtained when five strains of *Bact. tularense* were tested against streptomycin and aureomycin. As can be seen, there is no correlation between the degree of virulence and the susceptibility to the drugs. Sensitivity or resistance to antibiotics is thus no criterion of virulence of *Bact. tularense.*

Antigen yield from strains of different virulence.—The antigen precipitation titers of 1 ml of each of the four successive extracts from 50 ml of a 1% suspension of acetone-dried organisms were multiplied by 50 to give the number of antigen units in each 50 ml preparation. A unit of antigen was defined numerically as the reciprocal of the dilution at the precipitation titration end point. Thus a preparation with an end point at the 1:4 dilution contained four antigen units per ml. The total antigen units extracted was the sum of the antigen units contained in the four successive extractions. The results of the calculations are given in table 6. Twice the standard deviation of such precipitation titrations was 0.43 log units.[13] A calculation of the standard error of the difference ($SE_{diff} = 2\ SD\sqrt{2}$) indicates that there was no significant difference (95% probability) in the amount of antigen extracted from strains Schu, Nevada 14, and 425-F_4G, but that significantly less (95% probability) was extracted from an equivalent weight of strain 38 organisms.

The yield of antigen units of Schu, Nevada 14, and 425-F_4G per Blake bottle was 8.5 to 10.9 times that of strain 38, when both antigen precipitation titer and dry weight were taken into account.

Since it has previously been established that the degree of mouse protection is proportional to the amount of antigen used to immunize the mice,[11] these data indicate that the strain 38 extracts contained significantly less

TABLE 5.—*Susceptibility of 5 strains of* Bact. tularense *of varying degrees of virulence to streptomycin and aureomycin.*

Strain*	Size of zones of inhibition, in mm	
	Streptomycin	Aureomycin
	10 μg	10 μg
Schu	33	18
Nevada 14	39	35
425-F_4G	37	36
41804A-rough	37	25
38	47	30

* Listed in decreasing order of virulence.

TABLE 6.—*The yield in dry weight and antigen content of four strains of* Bact. tularense.

Strain*	Yield per Blake bottle	Antigen units per gg dry weight organisms
Schu	0.20 g	254,400
Nevada 14	0.31 g	160,000
425-F_4G	0.30 g	217,600
38	0.11 g	59,200

* Listed in decreasing order of virulence.

13. Ormsbee, R. A. Precision of measurement of LD_{50} end points in mice challenged with *Bacterium tularense.* J. Immunol. In press.

protective antigen. The other three strains did not differ among themselves significantly in this respect.

The residues remaining after the four successive extractions were titrated by the capillary-tube precipitation technic. The end point titers were Schu, 1:256; Nevada 14, 1:128; 425-F_4G, 1:256; 38, 1:128. The significance of these figures in terms of protective antigen is obscure, since the antigen-antibody reaction was neither a precipitation reaction nor an agglutination reaction as these terms are commonly used. They were essentially flocculation reactions of antibody with fragments of bacterial cells comprised both of cell-wall antigen and of internal constituents. An attempt was made, therefore, to assay their protective antigen content by the mouse protection test. The results are given in table 7.

If the results from similar protection tests employing antigen released by the ether treatment can be used as a basis of comparison,[13] the variation at both the 1:128 level and the 1:4 level is within the 95% probability range. Ormsbee and Larson[14] reported an average protective value of 2.28 (SD $\pm$ 1.63) for antigen of titer 1:4, and a corresponding value of 4.99 (SD $\pm$ 1.06) for an antigen of titer 1:256. It may be concluded tentatively that no significant strain differences in protective antigen content existed among the cellular residues remaining after four extractions.

TABLE 7.—*The mouse protective value of* Bact. tularense *residues after four extractions.*

Group	Antigen	Antigen precipitation titer	LD_{50} titer	Protection
A-1	Schu	4	4.87	3.6
B-1	Nevada 14	4	7.00	1.4
C-1	425-F_4G	4	6.00	2.4
D-1	38	4	6.71	1.7
A-2	Schu	128	2.50	5.9
B-2	Nevada 14	128	2.28	6.2
C-2	425-F_4G	128	2.38	6.1
D-2	38	128	1.53	6.9
F-1	None	−	8.44	—
F-2	None	−		—

DISCUSSION

Some of the criteria of virulence evaluated in the present paper can be used to distinguish rough strains of *Bact. tularense* from smooth strains and, since frankly rough strains are avirulent, they are valid tests for this purpose. However, none of these criteria can be used to distinguish between the virulent and avirulent smooth strains or to assess the degree of virulence among the smooth strains.

Also, the use of mice alone as in vivo controls in the in vitro virulence tests is insufficient. Since one viable organism of a strain of even low virulence will kill a mouse, only a small part of the total spectrum of virulence will be demonstrated by the use of this animal.

Observation of the colonial morphology of strains of *Bact. tularense* on CGA will likewise detect the frankly rough strains, but, as pointed out by Eigelsbach, Braun, and Herring,[4] "both smooth and nonsmooth variants of strain 38 proved to be avirulent" and, therefore, there may be "independent variation between virulence [and] colonial morphology." In the experiments reported here, all strains tested, except the frankly rough 41084A, yielded the smooth type of colony. Differences in colony size and rates of growth were noted but these varied considerably with the age or lot of medium and cannot be considered as reliable guides.

Studies of the electrokinetic reactions of the cell surfaces will likewise distinguish rough from smooth strains of *Bact. tularense* but will neither assess

14. Ormsbee, R. A. and Larson, C. L. 1955, Studies on *Bacterium tularense* antigens. II. Chemical and physical characteristics of protective antigen preparations. J. Immunol. **74**: 359–370.

the degree of virulence nor detect the avirulent smooth strains. Avi-Dor and Yaniv[6] found that the stability of suspensions of virulent strains was not influenced by low concentrations of several electrolytes and dyes, whereas avirulent strains, some of which were derived from the virulent strains, showed practically complete agglutinability under the given experimental conditions. The "highly virulent" strains were stated to have an LD_{50} of 10^{-10} and the low virulence strains of 10^{0} to $10^{-2.5}$, the figures representing dilutions of standards containing 10^{10} organisms per ml,[6] but the amount injected, species of animal used for test, and route of infection were not given. Eigelsbach, Braun, and Herring[4] found that in some cases acid or acriflavine agglutination was better correlated with virulence than was colony type, but, here again, the number of organisms necessary to kill mice when injected by the intraperitoneal route was used to designate virulence. In the experiments reported here, the frankly rough avirulent 41804A strain and the smooth avirulent Jap S3 strain were agglutinated by the *N*/160 sodium chloride method and only 41804A by the acriflavine method. In both cases the controls were also agglutinated. Suspensions of other avirulent strains were as stable as virulent strains by these tests. Thus, these methods cannot be used as criteria of the degree of virulence of an unknown strain.

It has been suggested by Eigelsbach, Braun, and Herring[4] and by Yaniv and Avi-Dor[7] that avirulent types of *Bact. tularense* arise through overgrowth of the virulent parent members of the clone by spontaneously arising avirulent variants. The former authors stated that "conditions adverse to the parent type are reached in all growing cultures and are apparently due to the accumulation of one or more toxic factors." They found that an avirulent strain (strain 38) had a higher percentage of variants than did a virulent strain (Schu) after a given period of incubation and thought that more rapid metabolite production by strain 38 could possibly have accounted for the observed differences. Yaniv and Avi-Dor[7] found that growth of avirulent strains suppressed that of virulent strains " . . . particularly if seeding of the virulent type was delayed until growth of the avirulent type had been established." They inferred that the avirulent type excreted substances inhibitory to the growth of the parent virulent strain and presented a photograph of a plate in which growth of a virulent strain was apparently severely inhibited by the close proximity of the avirulent culture growth. If this were a universal phenomenon associated with the degree of virulence, it should be possible to compare each new strain with several standard strains of known virulence and thus evaluate the virulence of strains.

We have endeavored to demonstrate overgrowth of virulent by avirulent variants derived from the same strain or in mixtures of virulent and avirulent strains. The procedure of Yaniv and Avi-Dor was duplicated in an attempt to repeat their demonstration of inhibition correlated with the relative virulence. Our results, in contrast to those of Yaniv and Avi-Dor, indicated that inhibition by the homologous strain as well as by heterologous strains could be demonstrated, but that such inhibition was completely independent of the relative virulence of the strains employed. The one exception concerned strain 38. With this relatively slow-growing strain, neither homologous nor heterologous inhibition could be demonstrated within the experimental period.

These data furnish no evidence for the production by avirulent strains of a factor which was inhibitory for virulent strains, in contrast to the data of Yaniv and Avi-Dor. The particular virulent strains employed simply overgrew the avirulent.

However, in the experiment in which five strains of *Bact. tularense* were subcultured serially on egg-yolk medium, marked reduction of virulence occurred in all of the strains tested. This suggests that, on this medium, overgrowth of the virulent by avirulent variants has occurred, but the role of the substrate in this reduction of virulence would seem to be important, since, in our hands, the same method employing a different medium (CGBA) failed to cause any loss of virulence after a far greater number of subcultures.

From these data, it would not seem probable that overgrowth of virulent by avirulent variants is a common mechanism by which the virulence of *Bact. tularense* is altered.

Yaniv, Avi-Dor, and Olitsky[6] have remarked that "most of the [streptomycin] resistant strains showed a greatly decreased virulence and a connection between these two properties appears likely." However, they tested streptomycin-resistant and nonresistant strains for susceptibility to aureomycin and chloramphenicol but no cross-resistance was found. It is uncertain whether Yaniv et al believe that there is a connection between loss of virulence and development of streptomycin resistance only, or between loss of virulence and resistance to any (but not necessarily all) antibiotics. In our experiments avirulent strains may be fully as susceptible to the action of these drugs as are the virulent.

If it could be established that, as indicated by the results obtained by Eigelsbach, Braun, and Herring[8] and Downs and Woodward,[9] there is "a close relationship between the virulence and immunogenic potency" of strains of *Bact. tularense*, immunogenicity could be a theoretical, if not a practical, method of measuring virulence. However, we have been unable to demonstrate any significant difference between the yield of antigen or the protective activity of two highly virulent (Schu and Nevada 14) and one relatively avirulent (425-F_4G) strains of *Bact. tularense*. The avirulent strain 38 gave a significantly lower yield than the three abovementioned strains. This lower yield of antigen appeared to be a reflection not only of its slower growth rate but also of the quantity of antigen present in each organism. There was, however, no difference in the degree of protection afforded mice by equivalent amounts of antigen from the different strains.

The results of the studies presented here have led us to the conclusion that, while the described methods may, on occasion, serve as valuable adjuncts for the characterization of a strain, the method of choice for the designation of virulence is the previously described method,[1] i.e., injection of animal species of varying degrees of susceptibility to *Bact. tularense* and calculation of the number of organisms of the given strain necessary to kill each species.

SUMMARY AND CONCLUSIONS

1. Various criteria frequently used to distinguish virulent and avirulent strains of *Bacterium tularense* have been tested against strains of known virulence as tested by a standard procedure.

2. Colonial morphology and electrokinetic cell-surface reactions will distinguish rough- from smooth-reacting types but do not distinguish virulent and avirulent smooth types nor assess the degree of virulence of the virulent strains.

3. Loss of virulence of *Bact. tularense* on artificial mediums could be demonstrated only on one medium. In other experiments in which mixtures of virulent and avirulent strains were used, the virulent strains dominated the avirulent, probably due to the far greater growth rates of the former. The contrary findings of others is, thus, not a general phenomenon and, therefore, not a valid criterion of virulence.

4. In studies of immunogenic potency, the avirulent strain 38 possessed a significantly lower protective antigen content than the more virulent strains. However, the protection afforded per unit of antigen was the same with all four strains employed.

5. It is concluded that the most valuable criterion of virulence of strains of *Bact. tularense* is injection of animals of various susceptibilities and calculation of numbers of organisms necessary to kill each species tested.

STUDIES ON THE PASSAGE OF HELMINTH LARVAE THROUGH HOST TISSUES

III. THE EFFECTS OF *TAENIA TAENIAEFORMIS* ON THE RAT LIVER AS SHOWN BY HISTOCHEMICAL TECHNIQUES

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Studies of penetration of the skin by various helminth larvae have shown that the larvae of some species impose extensive alterations on the basement membrane and ground substance of the host dermis.[1] We have proposed that the observed changes are caused by the production of collagenase-like enzymes by the larvae and that these enzymes are associated with the mechanism of penetration. However, not all of the species studied possess these collagenase-like enzymes and presumably some species employ different mechanisms of tissue penetration. The present investigation was instituted to determine whether helminths invading tissues other than the skin cause changes in host glycoprotein* similar to the changes imposed by the skin-penetrating larvae. This is of particular interest since any helminth migrating through or growing in tissue must necessarily traverse basement membranes or other areas of acellular glycoprotein which could conceivably constitute important barriers to their progress and development. The migration and early development of the strobilocercus of *Taenia taeniaeformis* was studied, as its course is relatively easy to follow during the first few days to several weeks of infection. Also, in this early period of its development the parasite is growing at its maximum rate. Histological studies have been made of the liver changes occurring during this period which suggest that substances produced by the larvae stimulate mitosis in the liver cells around the site of infection.[2] Equally important to the present investigation is the fact that there is a considerable body of information on the normal state of glycoprotein and other constituents of the rat liver derived from studies utilizing the freezing-drying method of fixation and the Hotchkiss and Evans blue staining techniques.[3,4] A preliminary report of

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* For convenience, the terms polysaccharide-containing protein and glycoprotein are used synonymously throughout the paper to refer to the Hotchkiss-positive substances not removed by treatment with saliva or methyl alcohol-chloroform.

2. Bullock, F. D. and Curtis, M. R. 1924, A study of the reactions of the tissues of the rat's liver to the larvae of *Taenia crassicollis* and the histogenesis of cysticercus sarcoma. J. Cancer Res. **8**: 446–481.
3. Aterman, K. 1952, Some local factors in the restoration of the rat's liver after partial hepatectomy. I. Glycogen; the Golgi apparatus; Sinusoidal cells; the Basement membrane of the sinusoids. Arch. Path. **53**: 197–208.
4. Aterman, K. 1952, Some local factors in the restoration of the rat's liver after partial hepatectomy. II. "Watery vacuolation": its relation to the vacuolation of anoxia. Arch. Path. **53**: 209–216.

the results of our investigation of *T. taeniaeformis* has been made.[5]

MATERIALS AND METHODS

A series of 40 female 150 g white rats (Holtzman) were infected via stomach tube with eggs obtained from gravid proglottids of *T. taeniaeformis* recovered at necropsy of experimentally infected cats. Each rat received 250 to 300 eggs and individuals were killed at intervals varying from 15 minutes to 20 days after infection. Portions of the intestine and liver were fixed by the freezing-drying method. Polysaccharide-containing protein was studied by means of the Hotchkiss technique, which utilizes the periodic acid-leucofuchsin reagent. The injection of Evans blue prior to fixation by freezing-drying was used to determine the presence and distribution of water-soluble, alcohol-insoluble polysaccharide-containing protein. Toluidine blue staining to demonstrate metachromatic changes was also employed. Relative amounts of soluble Hotchkiss-positive material were determined by comparing sections extracted with phosphate buffer and sections not so treated. Similarly, glycogen was determined by the difference between sections treated with buffered saliva and those not so treated prior to staining with the periodic acid-leucofuchsin reagent. The details of these methods, their usage and interpretation are fully discussed in an earlier study.[1] Animals on a normal regimen were studied to determine the state of the glycogen content of the liver while other animals were starved for 24 or 48 hours prior to autopsy to reduce the amount of glycogen in the hepatic cells. This was a convenient method of decreasing the Hotchkiss-positive glycogen which otherwise interfered with observations on changes in the liver glycoprotein.

It was found advantageous to use a phase microscope in studying the Evans blue preparations of *T. taeniaeformis* infections, particularly in those of 3 days duration or less. The earliest stages of the parasite could thus be located in the surrounding tissue. This is difficult with the usual light microscope since the tissues are cleared in Shillabers' immersion oil and neither the parasite nor the surrounding cells are stained.

Three helminth infections other than those mentioned above were also studied from more limited material and will be briefly discussed. These include *Hymenolepis nana* infections in white mice (CF 1) during the first 24 hours after infection via stomach tube, a single natural infection of a cat with the liver fluke *Amphimerus pseudofelineus*, and a 5-week infection of an arctic vole with *Echinococcus sibiricensis*.* The early stages of *Hymenolepis* in the intestinal villi were used for comparing with taenia larvae as they penetrate the intestinal mucosa. The limited material available on *Amphimerus* and *Echinococcus* provided information on liver changes for comparison with the findings in the taenia infections.

Tests on the collagenase activity of *T. taeniaeformis* were conducted in the manner previously reported,[1] using an azocoll substrate in *M*/10 phosphate buffer at pH 6.5 and were incubated at 37 C. for periods varying from 1 to 16 hours. Penicillin and streptomycin were included in the system. The wet weights of the various substances tested were essentially equivalent before extraction. Cysticerci from 16- and 100-day infections, mature and immature proglottids of adult *T. taeniaeformis*, and liver from both infected and normal animals were tested. Liver samples from infected animals were taken from the immediate vicinity of the larvae. The cysticerci were freed of liver tissue before homogenization. The helminth samples and the tissues tested were extracted by homogenizing in sterile normal saline and centrifuging at 18,000 RCF for 1 hour at 4 C.

RESULTS

The liver of uninfected control rats which had been injected with Evans blue 15 minutes prior to decapitation showed no prominent concentrations of dye. However, enough of this dye was retained in the vessels to give the entire liver a blue tinge. In sections no cells were stained and dye was prominent only in the lumina of the larger blood vessels. Essentially none was retained in the hepatic sinusoids between liver cords. The elastica interna of the larger vessels was lightly stained. In the liver from animals on a normal diet large amounts of glycogen were present in the hepatic cells. Frozen-dried sections stained by the Hotchkiss method

5. Lewert, R. M. and Lee, C. L. 1953, The effects of *Taenia taeniaeformis* and *Amphimerus pseudofelineus* on the liver as shown by histochemical techniques. J. Parasitol. 39: 4, sec. 2.

* The authors wish to thank Dr. Robert Rausch of the Arctic Health Research Center for providing the echinococcus material.

showed glycogen granules evenly distributed within the cells and within the individual liver lobules. The amount of liver glycogen was greatly reduced in rats starved for 24 or 48 hours, but the extent of reduction varied from animal to animal. Typically, a light scattering of glycogen granules remained with more present in the cells at the center of the lobule than in those at the periphery. In these animals the basement membrane of the hepatic sinusoids could be seen in some places as a thin, faintly-stained line, lacking continuity. The sinusoidal cells in some areas contained small Hotchkiss-positive granules. The normal liver stained with toluidine blue exhibited metachromasia only of the intima of the blood vessels and of the connective tissue surrounding the larger veins.

Twenty-four hours after infection of rats with *T. taeniaeformis* the organisms were found in the liver midway between the periphery and center of the lobule. They were approximately 10×16 μ in size and could be positively identified by the larval hooklets which were readily visible with the phase-contrast microscope. Some of the individuals were seen to be surrounded by endothelial cells while around others no endothelium was visible. In Evans blue-injected animals, a small amount of dye was retained about the parasite. Its distribution was eccentric with respect to the parasite and a space 1 to 2 μ in width, filled with amorphous blue-staining material, separated the parasite from the adjacent hepatic cells. In some cases, several of the hepatic cells bordering the parasite were surrounded by faintly blue intercellular substance. At this time there was no evidence of leucocytic accumulations about the infection site. Following staining by the Hotchkiss method the parasites had a faint pink color and were almost entirely lacking in glycogen. This was in great contrast to the intense staining of the embryo within the egg. The hepatic cells immediately adjacent to the parasite had less glycogen than the liver cells nearby. In a few instances, a slight increase in intensity of staining with the periodic acid-leucofuchsin reagent and a thickening of the basement membrane of the hepatic sinusoid were visible.

Seventy-two hours after infection, the developing cysticercus was still a solid mass of cells and was more sharply outlined with dye-retaining amorphous material following the injection of Evans blue than at the earlier periods (see pl. 1, fig. 1). In some infections this material was dense and appeared to be a well defined blue acellular capsule a few microns in thickness. In others it was 10 to 15 μ thick and less dense peripherally or essentially fluid in nature as could be deduced from its content of ice crystal artifacts. At the periphery of this blue-retaining liquefaction area the liver cells appeared normal but slightly compressed to accommodate the growing larva. Material staining a pale diffuse blue extended between the liver cords about the parasite and was usually more extensive on one side of the larvae. In 72-hour infections of rats starved for 48 hours the larvae varied in size from 27 to 32 μ in maximum diameter. Staining with the periodic acid-leucofuchsin method revealed increases in intensity of staining and amount of the Hotchkiss-positive substances. The parasite at this time had some intracellular glycogen and was enclosed in a deeply staining acellular capsule of glycoprotein. A more lightly stained area containing ice crystal artifacts surrounded this. Between the liver cords in the vicinity of the parasite the hepatic sinusoids stained more prominently than

they did in areas not containing parasites, and the distribution of this Hotchkiss-positive material was identical to that of the material retaining the Evans blue. Peripheral to this layer of diffusely stained material were slightly compressed hepatic cells that were glycogen-free in either starved or unstarved rats. Some of the sinusoidal cells surrounding the parasite had more Hotchkiss-positive granules than normal. In sections treated with buffered saliva, parasite and liver glycogen were removed and the color of the acellular capsule of the parasite was considerably reduced. In addition, the basement membranes of the sinusoids in the vicinity of the parasite were most intensely stained and in some instances were thickened or swollen.

On the 4th day of infection (except for growth and development of the parasite) the differences as compared to the 3rd day were largely a matter of degree. At this time the site of the parasites in the liver could be determined by gross examination of the liver of Evans blue-injected animals. Those close to the liver surface were visible as minute blue dots. The 4-day-old cysticerci ranged from 33 to 55 μ in diameter and had a thin acellular capsule that stained deeply with both the Evans blue and the periodic acid-leucofuchsin reagent. The amount of glycogen increased in the parasites while more hepatic cells around the parasite were glycogen-free. Basement membranes of the adjacent sinusoids were more prominent than previously.

By the 5th day the parasites had grown to as much as 50×80 μ in size and each was surrounded by an evident zone of altered liver approximately 70×130 μ in maximum dimensions. A typical parasite from a rat starved for 24 hours was a hollow ovoid structure 42×81 μ, containing scattered irregular glycogen masses with its cells. It was enclosed by a thin acellular capsule which stained deeply with Evans blue and was also Hotchkiss-positive. The capsule was not metachromatic with toluidine blue. Surrounding the capsule was an area 15 μ thick which stained less intensely with Evans blue and the Hotchkiss method and which was metachromatic with toluidine blue. It sometimes contained ice crystal artifacts. Peripheral to this area was a 25 to 40 μ wide layer of hepatic cells which were slightly compressed and completely free of glycogen. Surrounding the glycogen-free layer of cells was a single or double layer of hepatic cells containing some glycogen while outside this area[1] the cells were again free of glycogen.

In unstarved rats the parasites were somewhat larger (80×80 μ) and each was surrounded by an area of apparently normal, slightly compressed liver cells which however were completely free of glycogen (see pl. 1, fig. 3 and 4). As in infections of shorter duration, the basement membranes of the nearby hepatic sinusoids appeared slightly thickened and more prominent than normal. In liver sections on the 5th day of infection and thereafter the parasites were occasionally surrounded by degenerating hepatic cells, free nuclei, leucocytes and debris.

During the next few days of parasite growth the liver changes already described continued and increased in extent. By the 8th day the parasite was a thin hollow spheroid 170×300 μ in dimensions and the surrounding area of altered liver was 50 to 100 μ wide (pl. 1, fig. 5). Within this area Evans blue was retained. The same area was diffusely stained by the Hotchkiss method and the entire area was metachromatic with toluidine blue. Alteration of the basement membranes of the adjacent hepatic sinusoids was more prominent than

before, as evidenced by increased staining and the sinusoidal cells contained more Hotchkiss-positive granules. By the 13th day the parasite measured up to 500 μ in diameter. The surrounding area of altered liver extended 100 to 200 μ on all sides of the organism. Beyond this, the liver cells were apparently normal in appearance except that they were depleted of glycogen (pl. 1, fig. 6). The altered area of liver was sometimes infiltrated by leucocytes. The acellular capsule enclosing the parasite still stained intensely by the Evans blue and the Hotchkiss methods. However, less Hotchkiss-positive material could be extracted from it by treatment with buffer or buffered saliva than could be removed from the earlier stages. The parasite itself possessed more intracellular glycogen than was seen in younger infections. The cells immediately surrounding the parasites were by this time extensively altered and compressed. Fibroblast-like cells containing granules of Hotchkiss-positive material were mixed with the altered hepatic cells. As in infections of shorter duration the entire altered area of liver which measured 2½ times the parasite diameter stained a diffuse blue with Evans blue, was diffusely pink when stained by the Hotchkiss method, and was metachromatic with toluidine blue. The metachromasia was most intense at the extreme periphery of the area of altered liver cells. This localization of the limit of the altered area corresponded to the peripheral limits observed using the other techniques.

At or near this time the increases in intensity of Hotchkiss staining and in thickness of the basement membranes of the hepatic sinusoids were maximal and the sinusoidal cells contained many more Hotchkiss-positive granules than before. The effect was such that following staining, the sinusoids in the immediate vicinity of the parasite contrasted strongly with the very pale sinusoids in near-by normal areas of the liver. In the altered areas, the hepatic cells were also more sharply delineated due to the fact that the intercellular material between the cells was more intensely stained than normal. These features are illustrated in plate 2 in which two portions of the same liver are shown, one from an unaltered area and another taken from the periphery of an area altered by infection.

Alterations of the liver such as those described could be found throughout the first month of infection during which the parasite was growing rapidly. In older infections, the parasite was surrounded by a well-defined capsule of connective tissue and few changes were observed in the surrounding liver. No Evans blue was retained in the liver about the parasite and the intensely Hotchkiss-positive acellular capsule surrounding the organism did not stain with Evans blue. The sinusoidal basement membranes and cells appeared normal.

No consistent gross changes were observed in the intestinal mucosa of rats infected with *T. taeniaeformis*. Animals infected for 15, 30 and 90 minutes and 2, 4 and 24 hours were injected with Evans blue 15 minutes prior to decapitation. Blue villi were sometimes found but could not be associated with the presence of penetrating larvae. The lymphatic nodules of the intestine and mesentery from the infected animals retained more Evans blue than those from the controls. In frozen-dried material, no concentrations of Evans blue were found associated with the penetrating larvae and no consistent changes in polysaccharide-containing protein were observed in material stained by the Hotchkiss technique. Similarly negative results were obtained in mice in-

fected with *Hymenolepis nana*. In the latter infection animals were also sacrificed at intervals ranging from 15 minutes to 24 hours after infection and the tissues treated in the same manner.

In the single 5-week infection of the vole with *Echinococcus* that was studied, there were some liver changes reminiscent of those observed in the taenia infections. Areas retaining Evans blue surrounding the parasite resembled the areas of liquefaction surrounding the *Taenia* larvae. However, there were no intensely stained acellular capsules immediately adjacent to the larvae. The infection was extremely heavy and only small islands of liver tissue remained. In these islands the basement membranes of the sinusoids were heavily stained by the Hotchkiss technique. No normal appearing liver was present. The single natural infection of the cat with *Amphimerus* which was studied also gave evidence of differences from normal with respect to the glycoprotein. In areas of hyperplastic biliary epithelium in the vicinity of the adult flukes, Evans blue was retained beneath the epithelium and in the connective tissue developing around the bile ducts. These areas also contained Hotchkiss-positive material which was soluble in buffer. No marked changes in the hepatic sinusoids were noted. As in the case of the echinococcus infection, no material was available for study of the earlier stages of infection.

Tests for collagenase activity were made on various stages of *T. taeniaeformis*, normal liver, and liver altered by infection. Young cysticerci were highly active against the azocoll substrate while older cysticerci were much less active. Proglottids of the adult cestode, liver removed from close proximity to cysticerci and liver from normal animals were uniformly negative. Similar results with respect to collagenase activity were obtained when either extracts or whole pieces of the helminth or tissue were incubated with the substrate. A summary of the results made on the various extracts is given in table 1. No quantitative determinations of dye release were made.

TABLE 1.—*Collagenase activity of* T. taeniaeformis *as shown by incubation of saline extracts with azocoll.**

Hours	1	2	4	16
16-day cysticercus	±	+	+	+++
100-day cysticercus	−	−	±	++
Gravid proglottid	−	−	−	−
Immature proglottid	−	−	−	−
Infected liver	−	−	−	−
Normal liver	−	−	−	−
Buffer control	−	−	−	−
Collagenase control	±	+	++	++++

* 6 mg azocoll: 0.5 ml 0.1 *M* phosphate buffer pH 6.5: 0.5 ml extract, incubated at 37 C.

DISCUSSION

In this investigation, we were primarily interested in the effects of the cysticercus of *T. taeniaeformis* on the liver of the host during and immediately subsequent to the period of initial invasion, i.e., during the period of most rapid parasite growth. This period roughly encompasses 20 days and precedes the time when the parasite is completely enclosed in host-produced connective tissue. Concomitant with the rapid growth of the larvae, alterations occur in the state of glycoprotein portions of the liver adjacent to the parasites. The changes in host glycoprotein shown by the histochemical methods employed indicated that these acellular substances became more soluble. One month or more after infection parasite growth was slower, the cysticercus was surrounded by a connective tissue capsule, and the acellular liver constituents external to this were normal in reaction.

These alterations which we have encountered in the liver glycoprotein in taenia infections correspond closely to the conditions observed during the

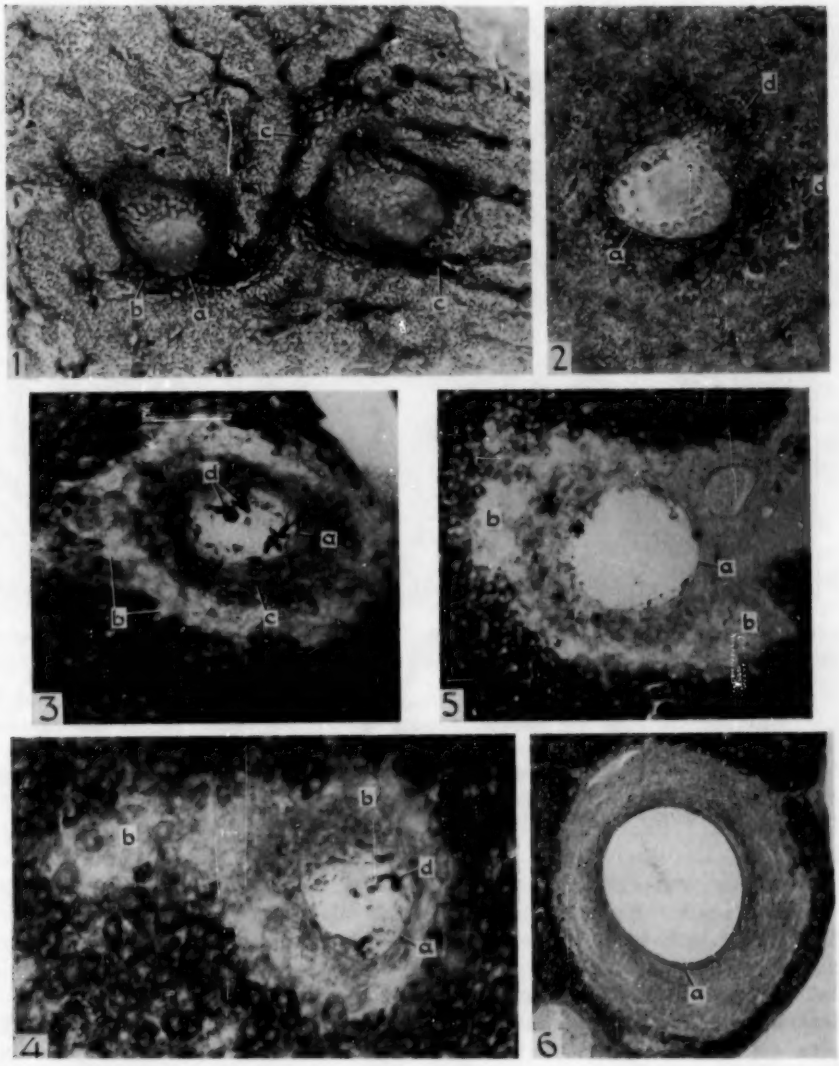

PLATE 1.—Rat liver sections with *T. taeniaeformis* cysticerci.

FIG. 1.—Section with two cysticerci, fixed by freezing and drying, from animal on third day of infection injected with Evans blue. ×475.

FIG. 2.—Similar to above but from fifth day of infection. ×210.

FIG. 1 and 2.—a, acellular capsule. b, liquefaction space with ice crystal artifacts. c, dye retained in sinusoids near parasites. d, peripheral limit of altered area retaining Evans blue.

FIG. 3–6.—Sections from unstarved rats with developing cysticerci. Fixed by freezing and drying and stained by the periodic acid—leucofuchsin method.

FIG. 3 and 4.—Fifth day of infection. ×210.

FIG. 5.—Eighth day of infection. ×135.

FIG. 6.—Thirteenth day of infection. ×55.

FIG. 3–6.—a, acellular capsule. b, liver cells normal in appearance but depleted of glycogen. c, compressed liver cells. d, parasite glycogen.

Note that in figures 3 to 6 the normal liver cells stain heavily due to their glycogen content. Their nuclei can be distinguished as lighter areas within the cells.

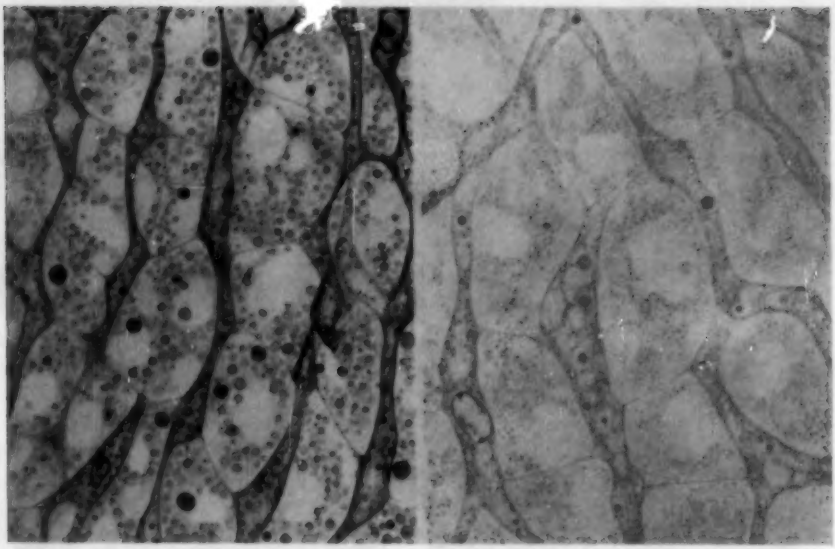

PLATE 2.—Camera lucida drawings of sections of normal and altered liver from the same animal on 8th day of infection, starved for 24 hours, fixed by freezing and drying and stained by the periodic acid—leucofuchsin method. Approximately ×1500.

Right: Normal portion of infected liver: The liver cells are free of glycogen and the liver cell granules are fine and relatively uniform in size. The sinusoidal cells are not prominent and the basement membranes are barely detectable.

Left: Portion from periphery of altered area near cysticercus. The liver cells are free of glycogen and have swollen granules. The sinusoidal cells are more prominent with darker staining granules than normal and the basement membrane is readily seen.

course of liver regeneration in rats following surgical removal of a part of the liver.[3,4] In the latter the basement membrane changes are interpreted as indicating that the glycoprotein is in a less polymerized state than normal. Evans blue retention is interpreted as indicating the presence of water soluble glycoprotein.[6]

It seems probable that the alteration of the liver is mediated by enzymes and is analogous to the alteration of dermal glycoprotein during penetration of the skin by certain helminths. The alterations were encountered during the period when the parasite is growing rapidly and displacing the surrounding tissue. At this same time, the parasite is presumed to produce some growth-stimulating substance, as evidenced by the increased mitotic activity around the site parasitized.[2] We think it significant that the maximal production of a collagenase-like substance by the larvae occurs at this time. Later in the infection when glycoprotein changes were reduced or inapparent, collagenase activity was also reduced. This collagenase-like enzyme produced by the larvae might act to soften intercellular materials and basement membranes, thus allowing cells to be displaced so as to more readily accommodate the rapidly

6. Gersh, I. and Catchpole, H. R. 1949, The organization of the ground substance and basement membrane and its significance in tissue injury, disease and growth. Am. J. Anat. **85**: 457–522.

growing larvae. Such an action might also increase the rate of diffusion of nutrient materials to the larvae as it renders its surroundings more fluid and permeable. It is interesting in this respect to note that the hepatic cells in the vicinity of the parasite were markedly lacking in glycogen while the parasites during early growth contain progressively more glycogen. The evidence that young parasites differ from later stages with respect to their secretions or metabolic products, in this case detected as a "collagenase," is of special interest. Campbell[7] has shown that antibody against the developing onchosphere differs from that produced later in the infection when the cysticercus is well established. This too indicates that the young parasite differs from the older cysticercus in its products or chemical constituents. In the absence of more direct evidence the possibility is suggested that this immunity toward young parasites may be directed against the "collagenase" activity of the larvae and thus negate or impair the invasive abilities of the onchosphere and/or developing cysticercus.

In our previous report the rapid changes caused in glycoproteins of the dermis by skin-penetrating helminths were related directly to parasite-produced secretions. The period of time elapsing between initial exposure and the first host-tissue changes was a matter of a few minutes or less. For this and other reasons, it appeared justifiable to assume that such early changes were due only to direct effects of the parasite on the host and not to an inflammatory response on the part of the host. In the taenia infections, however, the first definite alterations in the host tissue were not discernible until at least two days after initial infection. Therefore, the direct effects of the parasite could not be certainly differentiated from a possible inflammatory host response. It is certain that some of the glycoprotein changes encountered later were part of the usual host inflammatory response which includes formation of connective tissue as the host attempts to wall off the dedeveloping cysticercus. This formation of connective tissue following inflammation is similar to that discussed by other observers and does not merit special discussion here.

Alteration of the host glycoprotein from its normally polymerized state to a more fluid state would seem to be of advantage to other stages of *T. taeniaeformis* and to other parasites growing in or migrating through tissues. However, by our methods we were unable to demonstrate changes of this type in the small intestine of rats during the period when the mucosa was being penetrated by the onchospheres of *Taenia*. The results were also negative in the case of invasion of the intestinal mucosa of mice by larvae of *Hymenolepis nana*. Variation in the experimental conditions, however, may result in positive findings. Important factors may be the number and viability of the eggs used for infection, the movement and the contents of the intestine, and also the selection of an optimal time and location for removing samples. We believe that our findings indicate a need for further study rather than a conclusive absence of alterations in the intestine. The changes observed in the single specimen of vole liver infected with *Echinococcus* appeared to be similar in nature to those encountered in the taenia infections but early infections were not available for detailed study. Due to the extensive rapid liver inva-

7. Rausch, R. 1954, Studies on the helminth fauna of Alaska. XX. The histogenesis of the alveolar larva of echinococcus species. J. Infect. Dis. **94**: 178–186.

sion by this budding larval cestode,[8] it might be expected that even more marked changes would occur in the liver soon after infection. This parasite certainly merits further investigation by these methods. A similar statement can be made with respect to those infections with *Amphimerus* or with other liver flukes, in which biliary hyperplasia occurs in response to the presence of larvae. It would be of interest to determine whether this is associated with the action of parasite-produced enzymes on the acellular elements of the tissues.

SUMMARY

Infections of the rat with larvae of *Taenia taeniaeformis* were studied using histochemical methods to determine the state of acellular glycoprotein of the host liver. Tissues were prepared by freezing and drying and stained by the Hotchkiss method to show polysaccharide-containing protein and glycogen. Water soluble polysaccharide-containing protein was demonstrated in frozen-dried sections following the intravenous injection of Evans blue, and metachromatic changes were demonstrated by the use of toluidine blue. Qualitative tests for collagenase-like activity were performed using an azocoll substrate and extracts of cysticerci of various ages, portions of adult cestodes, normal and infected liver.

During the initial stages of infection and early growth of larvae of *T. taeniaeformis* in the rat, acellular glycoproteins of the liver are altered. These alterations include changes to a more soluble state and occur during a period when the larvae are producing a collagenase-like enzyme. Also during this period the hepatic cells surrounding the larvae are depleted of glycogen. When rapid growth ceases and the collagenase-like activity is reduced or absent, the larvae are surrounded by a well-defined connective tissue capsule and the adjacent liver is normal with respect to polysaccharide-containing protein and glycogen. The significance of the findings is discussed and compared to similar results in other helminth infections.

8. Campbell, D. H. 1938, The specific protective property of serum from rats infected with *Cysticercus crassicollis* J. Immunol. **35**: 195–204.

THE HISTOPATHOLOGY OF IMMUNE AND NORMAL MOUSE SKIN EXPOSED TO CERCARIAE OF *SCHISTOSOMATIUM DOUTHITTI* (TREMATODA: SCHISTOSOMATIDAE)

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CFI mice infected with *Schistosomatium douthitti*, a schistosome of muskrats and field mice in the United States, acquire resistance to reinfection 30 to 120 days after exposure. In immune mice challenged with an exposure of 50 cercariae only 27% of these cercariae develop into adult schistosomes, whereas 54% develop in control animals (Kagan, 1952). The following experiment was initiated to compare the inflammatory response between normal and immune mice exposed to cercariae of *S. douthitti*, and to determine if cercariae were immobilized in the skin of immune animals. A secondary objective was to determine whether the inflammatory response following exposure was caused by the cercariae or by contaminating bacteria introduced on the cuticle of the invading organism. Mice were exposed to normal "contaminated" cercariae and cercariae sterilized with antibiotics.

The earliest studies on the invasion of mammalian skin by schistosome cercariae were made by Japanese workers on *Schistosoma japonicum*. According to Faust and Meleney (1924), Miyagawa (1913) failed to find any cellular reaction surrounding cercariae in the skin of experimental animals. Narabayashi (1916) and Sueyasu (1920) found hemorrhage and cellular infiltration about cercariae in the skin of infected mice. Kakamato, in a personal communication, reported that cercariae placed on his forearm and excised after 2 hours showed no cellular reaction in the tissues. In their own studies Faust and Meleney (1924) exposed a dog to the cercariae of *S. japonicum* and 15 hours after exposure 2 pieces of skin were excised and 4 cercariae found in the dermis. Three of these cercariae were surrounded by a definite tissue reaction. In the skin of a mouse exposed to many cercariae and sectioned 24 hours after exposure there was no visible tissue reaction.

Miyagawa and Takemoto (1921) immersed young mice in water containing cercariae of *S. japonicum* and found after 24 to 48 hours that cutaneous reactions to the penetration of cercariae were practically nil, whereas inflammation in the lungs of these animals was very intense. Fairley and Mackie (1930) studied the penetration of *S. spindale* in the skin of goats and guinea pigs and found considerable local reaction occurring at the site of cercarial penetration. In the guinea pig 6 hours after exposure cercariae were surrounded by cells of inflammatory origin, and they suggest that in this animal the inflammatory response is the first line of defense. Watarai (1936) studied the cellular changes in the skin of normal and immune rats and chickens exposed to cercariae of *S. japonicum*. Watarai

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found that the cellular response varied with the number of cercariae used and in hyperimmune rats inflammatory cells appeared to be more aggressive in attacking cercariae, as though they were demonstrating some protective phenomenon. In the chickens he found that by the third day a majority of the cercariae had died in the skin. Koppisch (1937) studied the cellular response in the skin of rabbits and albino rats. According to Koppisch, Iturbe (1919) observed leucocytic infiltration in the skin of white rats, house rats, and guinea pigs exposed to the cercariae of *S. mansoni*. In the rabbits Koppisch found that the skin responded to the invasion of cercariae with congestion, edema and leucocytic infiltration. Cellular infiltration was evident 1 hour after exposure and consisted mainly of pseudoeosinophils and monocytes. In general the infiltration was diffuse until the 10th hour.

The cellular response in the skin of man caused by the penetration of human and nonhuman schistosome cercariae has been recently reviewed by Cort (1950). Evidence is given that schistosome dermatitis is a sensitization phenomenon with a mild cellular reaction following initial exposure and a more severe reaction after sensitization has taken place. Olivier and Weinstein (1953) reported that in the skin of rabbits sensitized against bird schistosomes the intense cellular response following re-exposure was associated with many dead and immobilized cercariae in the skin.

MATERIALS AND METHODS

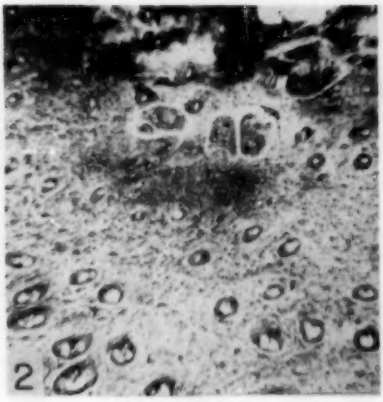
The abdominal skin of 21 CFI (Carworth Farms) mice was studied. Sections were obtained from 9 control mice 1, 2, 3, 7, 13, 24, 50, 70, and 80 hours after exposure and from 12 mice immunized by previous infection ½, 1, 2, 3, 4, 5, 13, 23, 35, 70, and 96 hours after exposure.

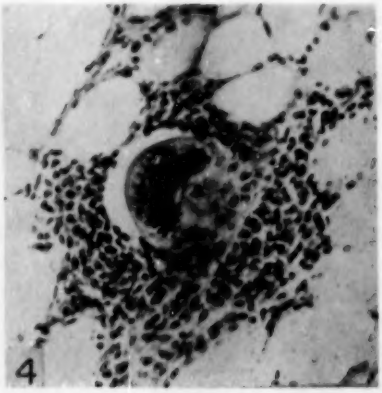
Mice were restrained 24 hours before an experiment and a small area of the abdomen was either carefully shaved, depilated with a commercial depilatory, or clipped closely with an animal clipper. At the time of exposure small circles in India ink were marked on the abdomen and cercariae were placed within these circles with a wire loop at hourly intervals (Kagan, 1952). Approximately 100 cercariae were used per area although in some mice 50 to 500 cercariae were used. Mice were killed by a blow on the head, the abdominal skin excised, stretched over a piece of cork with cactus needles and fixed for 5 hours in Helley's-Maximow's Zenker-formal (9 parts Zenker's solution without acetic and 1 part neutral formalin). Tissues were embedded in celloidin, sectioned at 10 μ parallel to the surface and stained with dilute Delafield's hematoxylin and counterstained with eosine and azure II. One series was embedded in paraffin and cross-sectioned serially.

Sterile cercariae were obtained by placing clean washed snails under a sterile screen in a jar with sterile water containing 1000 units of penicillin G plus 0.05 mg of streptomycin sulfate per cc of water for approximately 7 hours. Sterility was ascertained by inoculating tubes of veal broth and tubes of thioglycolate medium with cercariae shed by the snail, and incubating at 37 C. for several days. The skin of the mouse was sterilized by swabbing with ether several minutes prior to exposure. One mouse exposed to 21 sterile cercariae harbored 7 male and 4 female worms at autopsy, indicating that the cercariae do not lose their viability and infectivity under the conditions of the experiment.

EXPERIMENTAL RESULTS

Normal mice.—Seven mice were exposed to normal "contaminated" cercariae. One hour after exposure cercariae were found in the epidermis in tunnels parallel to the surface of the skin (fig. 1). Some cercariae had already penetrated into the dermis. Two hours after exposure many cercariae were still in the epidermis and moderate infiltration of heterophils was observed in the dermis. In one mouse autopsied 3 hours after exposure (fig. 2) inflammatory cells were collecting around cercariae. Seven hours after exposure cercariae were still in the epidermis. Inflammatory cells were seen diffusely scattered in the dermis and were not localizing





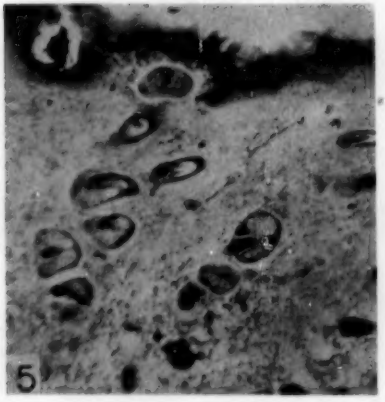


FIG. 1–4.—Penetration of cercariae of *S. douthitti* in normal mice.

FIG. 1.—Cercariae in epidermis 1 hour after exposure.

FIG. 2.—Three hours after exposure surrounded in the dermis by heterophils.

FIG. 3.—Seven hours after exposure. Note inflammatory cells in adjacent areas.

FIG. 4.—50 hours in subdermal fat. Note small mononuclear cells surrounding cercariae.

FIG. 5.—Sterile cercariae 13 hours after exposure in control animal. Note absence of inflammatory response.

FIG. 6.—Sterile cercariae 70 hours after exposure in blood vessel surrounded by erythrocytes.

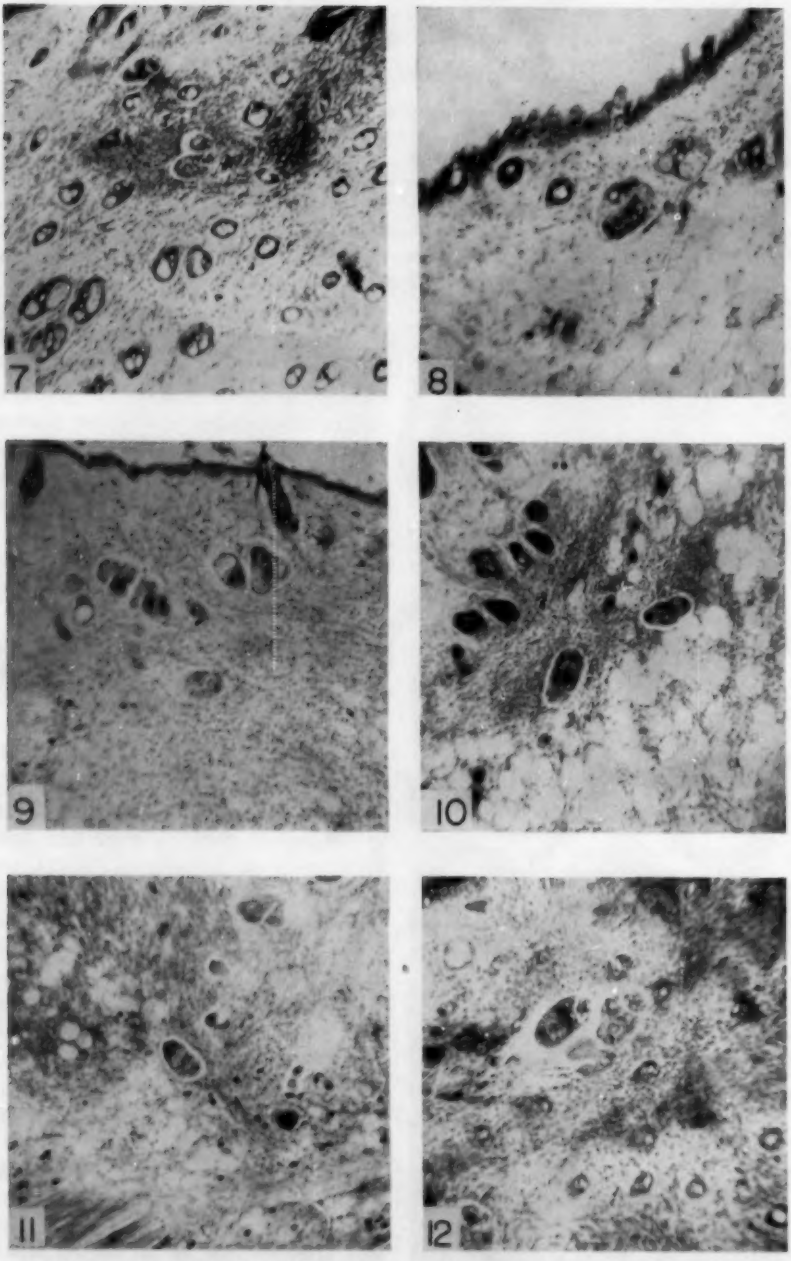


FIG. 7–11.—Penetration of cercariae in immune mice.

FIG. 7.—Normal cercariae 2 hours after exposure, surrounded by inflammatory cells.

FIG. 8–12.—Sterile cercariae employed for penetration.

FIG. 8.—Cercariae half hour after exposure in sebaceous gland.

FIG. 9.—Thirteen hours after exposure in dermis. Note absence of inflammatory response around cercariae.

FIG. 10.—Twenty-four hours after exposure. Note intense inflammatory response around cercariae.

FIG. 11.—Cercariae 70 hours after exposure, surrounded by inflammatory cells.

FIG. 12.—Cercariae in epidermis with tail attached.

around cercariae (fig. 3). Cercariae in the dermis 24 hours after exposure were in areas with many inflammatory cells but these cells were not attracted to the cercariae. After 50 hours cercariae were no longer present in the epidermis. In the dermal fat a cercaria was found surrounded by many mononuclear cells whereas the adjacent areas were free of cells (fig. 4). Eighty hours after exposure no cercariae were observed and the inflammatory response in the tissues was very mild.

Two normal mice were exposed to sterile cercariae and autopsied 13 and 70 hours after exposure. In these preparations the inflammatory response was mild and inflammatory cells were not attracted to cercariae (fig. 5). One cercaria 70 hours after penetration was found in a blood vessel (fig. 6).

The inflammatory response varied considerably between animals and may have been influenced by the method of preparing the skin, and the condition of the invading cercariae. Skin treated with a depilatory showed diffuse infiltration of heterophils in the dermis soon after exposure. Clipping the skin with animal clippers was the least irritating and this method was adopted for all mice in the immune series and a majority of the control animals. With the exception of one animal in this series (mouse autopsied 3 hours after exposure), the inflammatory process in the skin was neither marked nor localized.

Immune mice.—Two immune mice were exposed to normal "contaminated" cercariae. One mouse was infected with male worms and the second with male and female worms. In the former the inflammatory response was mild and inflammatory cells were not attractive to cercariae. In the mouse infected with male and female worms the inflammatory response was intense and many heterophils were in the dermis surrounding cercariae as early as 2 hours after exposure (fig. 7).

Immune mice exposed to sterile cercariae.—Ten mice immunized by previous exposure 30 to 60 days before re-exposure were autopsied. Thirty minutes after exposure cercariae were found penetrating into the dermis. Some were found in hair follicles and sebaceous glands (fig. 8), but the majority were in the epidermis. One, two, three, and 13 hours after exposure the inflammatory response was mild and cercariae were not irritating (fig. 9). Approximately 3 hours after exposure the number of heterophils in the dermis began to increase, building up to an intense heterophil response such that at 23 and 36 hours of exposure many cercariae in the dermis were surrounded by these cells as well as some fibroblasts and a few eosinophils (fig. 10). In areas adjacent to penetrating cercariae mobilization of fibroblasts was evident with some of these connective tissue cells lying close to the cercariae. At 70 hours after exposure cercariae were found penetrating parallel to the muscle of the mouse body wall, surrounded by many small mononuclear inflammatory cells (fig. 11). At 96 hours after exposure no cercariae were found and the sections showed very little inflammation.

DISCUSSION

The inflammatory response after exposure to cercariae in both normal and immune mice was predominantly heterophilic in character. The principal differences between immune and nonimmune animals was the intensity, rate of development, and focalization of the heterophil response. In immune mice the inflammatory response was more intense and developed earlier. Mobilization of fibroblasts in the dermis of immune mice developed approximately 24 to 48 hours after exposure. In normal

mice there was no evidence of such mobilization. There was evidence, however, in normal mice, of focalization of inflammatory cells at 50 hours after exposure and perhaps the beginning of immobilization of cercariae in the subcutaneous fat (fig. 4). The mobilization of fibroblasts and focalization of mononuclear cells around cercariae in the immune animal probably represents the extent of the tissue barrier in the skin invoked by the host.

Relatively large numbers of cercariae were used on a small area of skin. The penetration of cercariae and passage through the skin is fairly rapid for in sections made after 3 hours of exposure comparatively few cercariae were found.

Observations on the cellular response in the skin of rats after exposure to larvae of *Nippostrongylus muris* (Taliaferro and Sarles, 1939) and in the intestine of mice infected with *Trichinella spiralis* (Larsh and Race, 1954) have been made. In nonimmune rats the inflammatory response to nippostrongylus larvae was initiated 4 to 8 hours after penetration, whereas in immune rats the response was more intense and was initiated in 2 hours. With *T. spiralis* the difference in the inflammatory response in the intestine of immune and nonimmune mice was one of time and severity. In the nonimmune animal there was little to no inflammation for 4 days after penetration, whereas in immune mice inflammation was noted at 12 hours and reached its peak by 4 days. The inflammatory response in the skin of immune and normal mice exposed to schistosome cercariae follows a similar pattern of developing earlier and focalizing in immune animals and being diffuse in normal skin.

By exploiting the synergistic action of penicillin G and streptomycin sulfate in sterilizing living cercariae we have been able to ascertain whether the heterophil response is initiated by the invading cercariae or by contaminating organisms introduced in the train of penetration. The heterophil response following exposure to sterile cercariae was milder than with the use of contaminated cercariae. The fact that mobilization of heterophils developed several hours after exposure to these sterile cercariae suggests strongly that the stimulus for their appearance were the cercariae or cercarial products and not foreign contamination introduced in the skin.

Our observations paralleled many of the observations made by recent British workers on the route of penetration and penetration characteristics of cercariae of *Schistosoma mansoni* in the skin of mice (Gordon and Griffiths, 1951; Griffiths, 1953; Standen, 1953). With *S. douthitti* we, too, observed cercariae both in the epidermis (fig. 12) and in hair follicles with attached tails. The penetration route in mouse skin as outlined by Standen (1953) is essentially similar for cercariae of *S. douthitti*. However, we made no attempt to determine whether cercariae used the lymphatic system or small venules in leaving the skin. We did made an observation (fig. 6) of what is apparently a cercariae either in a capillary or penetrating through the capillary.

SUMMARY AND CONCLUSIONS

The inflammatory response in the abdominal skin of immune and normal mice following exposure to cercariae of *Schistosomatium douthitti* was studied.

The inflammatory response after exposure was predominatly heterophilic in character. In immune mice the rate of development and focalization of heterophils was more intense and developed earlier than in normal mice. There was evidence of focalization of small mononuclear cells around cer-

cariae and perhaps the beginning of immobilization of cercariae in the dermis of immune mice after 36 hours of exposure.

Cercariae sterilized with antibiotics have been introduced in the study of the inflammatory response following exposure to schistosome cercariae. The heterophil response following exposure to sterile cercariae was milder than after use of normal cercariae. The mobilization and focalization of heterophils and small mononuclear inflammatory cells around sterile cercariae indicates that the stimulus for their appearance must be the cercariae or some cercarial product and not foreign contaminants introduced in the train of penetration.

The cellular response in the skin of immune mice is such that we must conclude that the cercariae of *Schistosomatium douthitti* are not impeded or completely immobilized in their penetration of the skin.

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AN ANTIGEN PREPARED IN VITRO EFFECTIVE FOR IMMUNIZATION AGAINST ANTHRAX

I. PREPARATION AND EVALUATION OF THE CRUDE PROTECTIVE ANTIGEN

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Watson, Cromartie et al[1,2] noted two distinct and separable factors in extracts of the peculiar lesions found in rabbits that had died of anthrax—(a) an "inflammatory factor" which caused increased permeability of the tissues and hence presumably would aid in the spread of the infection throughout the animal body, and (b) an antigenic substance which successfully induced protective immunization against anthrax in other animals into which it was injected. The protective antigen was demonstrated by Gladstone[3] in the cell-free filtrate of cultures of *Bacillus anthracis* grown in vitro in a medium consisting principally of blood plasma or blood serum. Although this organism can be cultivated on almost all of the ordinary bacteriological culture mediums, Heckly, Goldwasser and Kefauver[4] reported that the albumin and the dialyzable portion of the serum are the essential constituents of the culture medium for the production of the protective antigen. Gladstone[3] further claimed that the essential dialyzable factor of the serum is sodium bicarbonate.

The object of the study described in this report was the development of a more practical method for the production of the protective antigen which is elaborated by the cultivation of *B. anthracis* in a suitable medium. The ultimate product would necessarily be made easily available in large quantity and in concentrated form and be efficient in the immunization of man or domestic animals against anthrax, with the minimum of risk of untoward effects, such as their sensitization to foreign proteins.

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2. Watson, D. W., Cromartie, W. J., Bloom, W. L., Kegeles, G. and Heckly, R. J. 1947, J. Infect. Dis. **80**: 28–40.
3. Gladstone, G. P. 1946, Brit. J. Exper. Path. **27**: 394–410.
4. Heckley, R. J., Goldwasser, E. and Kefauver, D. 1949, J. Infect. Dis. **84**: 92–97.

EXPERIMENTAL

The bacteriological culture medium

A medium was sought which would support more uniformly a satisfactory growth of *B. anthracis* with a concomitant high yield of protective antigen practical for the immunization of man and domestic animals. The culture medium that was employed in the preliminary work was that described by Heckly et al,[4] which was a 3.0% solution of sheep blood serum albumin in a commercially prepared serum ultrafiltrate.* *B. anthracis* was not easily adapted to this medium and a satisfactorily luxuriant growth did not always develop. At least one of the batches of the commercially available blood ultrafiltrate was unsuitable for this use because of inhibition exerted by it on the growth of this organism. Following Gladstone's suggestion,[3] the blood ultrafiltrate was satisfactorily replaced by sodium bicarbonate in the quantity calculated to be present in the blood serum medium, approximately 0.25 g per 100 ml. Later, this amount of sodium bicarbonate or equimolecular equivalents of other soluble bicarbonates was doubled and the predictable yield of the protective antigen was thereby increased. Some protective antigen was

* The commercial blood serum ultrafiltrate available was under the label of Schering and Glatz, Inc., and was prepared from ox blood serum.

produced when this serum albumin was partially hydrolyzed with HCl, but replacement by egg albumin or milk albumin led to failure.

The bacterial multiplication apparently was limited to a greater degree when the albumin portion of the medium was prepared from human blood than when sheep or rabbit blood albumin was chosen. Obviously a heavier growth was an essential requirement for the efficient production of the protective antigen. Addition to this medium of small quantities of nutrient material such as dextrose and tryptic digest of casein, yeast extract or nutrient beef broth, or a combination of these, resulted in a more luxuriant bacterial growth and a larger yield of protective antigen, regardless of the source of the serum albumin. Of the many formulas studied, the following culture medium, 243 was finally chosen as the most satisfactory from the standpoint of (a) high productivity of stable protective antigen, and (b) low content of any protein foreign to the subject of immunization, which might possibly sensitize the recipient of the injections of the cell-free filtrate of the bacteriological culture. The composition of this medium per liter is as follows:

A. Serum albumin, 10.0 g (dry weight).—The albumin was prepared from blood serum by the alcohol precipitation method of Cohn et al.[5] Serum of a species of animal homologous with or genetically related to the recipient was satisfactory and advantageous because, by its use, addition of sensitizing antigen was avoided.

B. Yeast extract, approximately 5.5 g (dry weight) = 148.5 ml of the following preparation.—A large cell strain of bakers yeast (such as Fleischmann 20–40) suspended in 0.01 *N* HCl was stirred for 24 hours at 23 to 25 C, while pH 4.0 was maintained by the intermittent addition of suitable quantities of concentrated HCl. The yeast cells of such a strain were removed readily by filtration through a medium grade filter paper. The pH of the filtrate was brought to 11.0 by the addition of NaOH, and the precipitate thus formed was removed by filtration and discarded. The filtrate was neutralized with HCl. Further removal of protein was accomplished by treatment of this yeast extract with activated charcoal, without thereby reducing the yield of protective antigen, and then it was sterilized by autoclaving at 15 pounds pressure for 12 to 15 minutes.

C. Dextrose, 10.0 g.

D. Salts.—$Na_2HPO_4 \cdot 12\,H_2O$, 6.96 g; $NaH_2PO_4 \cdot H_2O$, 0.675 g; KCl, 3.48 g; NaCl, 3.57g; $NaHCO_3$, 5.0 g.

E. Phenol red, as pH indicator, 12.0 mg (3.0 ml of 0.4 % solution).—The medium, pH 7.2, was sterilized by filtration through a Seitz filter. For routine cultivation of *B. anthracis* 50.0 ml were transferred to a 12 ounce flat prescription bottle ("medical flat"), stoppered with a cotton plug, and just before use the medium was acidified to pH 6.6 to 6.8 by absorption of CO_2 gas.

Production of the antigen

The medium was inoculated with 35,000 spores of a strain of *B. anthracis* of medium or low virulence, such as CD-2, elsewhere designated "5-B," so chosen for safety, although any one of the five strains of various virulence that were tested was satisfactory. The bottle was stoppered with a cotton plug, sealed with parafilm to retain the high CO_2 content and at the same time maintain lowered oxygen tension, and placed horizontally in the incubator at 37 C. After static incubation for 16 or 17 hours the culture was centrifuged and the supernatant filtered through a Seitz filter to remove the bacterial cells.

The criterion for the production of the protective antigen was based entirely on the effectiveness of immunization of animals against anthrax; rabbits or guinea pigs were satisfactory subjects for evaluation of the antigen. Although many of the tests reported were duplicated on rabbits and guinea pigs, the rabbit was selected as the standard test animal. The cell-free filtrate, adjusted to pH 7.0 to 7.2 and placed in the refrigerator at 4 C, was stable for at least 3 months and longer when 0.1% formalin was added. Three intradermal injections of the cell-free filtrate, usually 1.0 ml or 0.1 ml, were made in rabbits* at 6-day intervals. This course of immunization was chosen as a standard procedure in order to obtain true comparisons of the different preparations of antigenic material, including that investigated by Watson and coworkers[1,2] and Heckly and coworkers,[4] rather than to accomplish the highest possible degree of immunization of the animal. The adequacy of the protective immunization was challenged 7 days later by intradermal administration of spores of the virulent strain *B. anthracis* CD-25. This strain has also been called "M-36" or "British M-36."

Usually all of those rabbits that received the 1.0 ml doses and most of those that received the 0.1 ml doses survived the challenge with 1200

5. Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M. and Taylor, H. L. 1946, J. Am. Chem. Soc., **68:** 459–474.

* Rabbits, usually 3½ to 4 pounds, were pure or cross breeds as obtainable on the open market. Variation in breed or weights from 2 to 8 pounds made no appreciable difference in the results.

spores of this strain, 1000 times the number that resulted in anthrax fatal to the untreated rabbit.

Effect of omission of the albumin from the culture medium

A luxuriant growth of *B. anthracis* occurred, and some protective antigen was produced when the serum albumin of the culture medium was replaced by globulin or any of several other proteins, or omitted altogether from the medium, which otherwise afforded an excellent yield of the antigen. Without the albumin in the medium, however, the quantity and stability of the demonstrable protective antigen in the cell-free filtrate was relatively insignificant. It was demonstrated only after process of concentration, such as dialysis and lyophilization;* fractionation methods, such as those discussed in a later report,[6] were found to be useful. Frozen solutions of the dialyzed concentrated material were less stable than the dried antigen. The addition of the albumin after the incubation period did not suffice for the production of the protective antigen. Evidently the serum albumin must be an ingredient of the culture medium during the incubation period in order to procure the highest yield of the stable antigen.

Variation of the temperature of incubation

A rather luxuriant growth of *B. anthracis* developed during cultivation in this medium under wide limits of temperature control. The optimal temperature of incubation for production of the protective antigen of high titer, however, appeared to be at or near 37 C. This conclusion was drawn from a few experiments performed to determine the relative effectiveness of immunization of rabbits with the cell-free filtrates of cultures grown at 32 C, 37 C and 42 C (table 1). The smaller (0.1 ml) doses of the filtrate of the culture incubated at 37 C and the larger (1.0 ml) doses of all three culture filtrates perfectly protected the rabbits from anthrax. Deaths occurred in the groups of animals injected with the smaller (0.1 ml) doses of the filtrates of those cultures incubated at 32 C and 42 C.

Variation of the size of inoculum and prolonged bacterial cultivation

Heckly et al[4] have reported confirmation of the finding of Gladstone[3] that the size of the inoculum was important in respect to the yield of the protective antigen. These workers used the same strain of *B. anthracis* as was used in the work herein reported, strain CD-2. This point has been checked many times during the course of this project and it has been found that considerable deviation from 35,000 ±5,000 spores per 50.0 ml medium always resulted in a diminution of the yield of protective anitigen, although more or less growth occurred within 16½ hours, the time chosen as the standard for these comparisons.

Gladstone[3] and Heckly et al[4] also reported that the protective antigen was not found in appreciable quantity before about 12 hours of cultivation of the bacteria but became optimal in 16 to

TABLE 1.—*The effect of the temperature of incubation on antigen production, as demonstrated by rabbit protective tests.*

Temperature of incubation	Ratio of rabbits surviving the challenge of 1000 MLD *B. anthracis* spores to the total number of rabbits challenged		
	Dose culture filtrate		Controls: no injection before challenge
	3–1.0 ml	3–0.1 ml	
42 C	8/8	2/8	0/8
37 C	8/8	8/8	
32 C	8/8	5/7	

* The term "lyophilization" refers to drying from the frozen state.

6. Boor, A. K. and Tresselt, H. B. 1955, J. Infect. Dis. 97: 203–206.

TABLE 2.—*Demonstration of the protective antigen formed in the cultures of* B. anthracis *incubated for various periods under atmospheric and lower oxygen tensions.*

Period of incubation	Grown under atmospheric oxygen tension in bottles, cotton plugged				Grown under oil (low oxygen tension)			
	Strain CD-2 3 doses*		Strain NPA 3 doses*		Strain CD-2 3 doses*		Strain NPA 3 doses	
	1.0 ml	0.1 ml	1.0 ml	0.1 ml	1.0 ml	0.1 ml	1.0 ml	0.1 ml
17 hours	8/8	5/8	6/6	1/8				
42 hours	4/8	3/8	2/7	1/8	3/3	2/3	4/4	2/4
5 days	0/7	0/7	2/8	1/8	2/3	2/4	3/4	3/4
8 days							3/4	2/4
10–11 days	0/4	0/4	0/4	0/4	3/4	2/4	3/4	0/4
18 days							0/3	0/3
21 days					0/3	0/4		

* Rabbits were given 3 interdermal injections of 1.0 ml or 0.1 ml of the cell-free filtrate at 6-day intervals. This was followed in 7 days by the challenge with 1000 MLD *B. anthracis* CD-25. The fractions indicate the ratio of the survivors to the total number challenged. In each experiment all of the control rabbits died.

18 hours and diminished rapidly a few hours thereafter. Proteolytic destruction of the protective antigen in the culture was presumed to be the cause of the deterioration evinced after the 20 hours incubation period. A mutant strain of *B. anthracis*, NPA, was isolated from the Vollum strain by Dr. George Wright[7] and was found by him to grow in serum protein medium without exhibiting an appreciable amount of proteolytic action as shown by a minimum increase in NPN. The filtrate of the 17 hours culture of strain NPA and of strain CD-2 yielded about the same quantity of protective antigen. The decrease in the protective antigen which had occurred after incubating the culture of strain NPA for 42 hours was much like that in a similar culture of strain CD-2, although a definite difference was noted at 5 days (table 2). Static cultivation in "medical flats" fitted with uncapped cotton plugs was used for this comparison.

A destructive force other than the proteolytic enzyme elaborated by the growing organism was sought. The following experiments were performed to test the degree of responsibility of oxidation for the deterioration of the protective antigen during the incubation of the culture.

7. Wright, G. G., Hedberg, M. A. and Feinberg, R. J. 1951, J. Exper. Med. **93**: 523–527.

The culture medium (100 ml) described above was placed in each of several 125 ml separatory funnels supplied with glass stopcocks and the necks were plugged with cotton. The medium was acidified with CO_2 gas and inoculated with 70,000 spores *B. anthracis*, the same inoculum per unit volume as was used for cultivation in air in the "medical flats." The surface was covered to about ½ inch depth with paraffin, melting point 36 C.* At various periods of incubation (table 2), portions of the culture were withdrawn from the separatory funnel, the bulk of the cells removed by centrifugation, and the supernatant filtered through a Seitz filter. Definite protection against anthrax was developed in rabbits by immunization with the filtrates of the cultures that were removed from the incubator even after 10 days incubation while protected from the oxygen of the atmosphere. This was a much longer period than that during which the antigen could be demonstrated when the *B. anthracis* was cultivated in the air; the rates of the deterioration of the protective antigen in the two strains CD-2 and NPA were similar. The results of these experiments

* Paraffin of the melting point slightly below the temperature of incubation, 37 C , was chosen in order to maintain fluidity while in the incubator and to permit easy separation as a solid at room temperature.

support the assumption that oxidation as well as proteolysis plays an important role in the disappearance of the protective antigen in a culture incubated under the usual oxygen tension.

Stabilization of the refrigerated cell-free culture filtrate by formaldehyde

The stability of the protective antigen in the refrigerated culture filtrate appears to be improved somewhat by the presence of formaldehyde. The cell-free filtrate of the static *B. anthracis* culture (248) was divided into two equal parts and placed in rubber stoppered serum vials. To one vial was added 1.0 ml neutral formalin per 100 ml. Neutrality was maintained by additions of dilute NaOH as seen to be necessary by observing the color change of the phenol red, a constituent of the culture medium. At intervals of a few weeks the titers of the protective antigen in the two samples were compared. After the preparations had remained in storage at 4 C for 30 weeks, rabbits were protected perfectly by three injections of 1.0 ml of the original culture filtrate (248) or 1.0 ml or 0.1 ml of that which contained formaldehyde (248F). However, at that time 0.1 ml doses of preparation 248 afforded only about one-third of the protection given by injections of the fresh preparation. The interference of the formaldehyde with both proteolysis and oxidation[8] must be taken into consideration in seeking an explanation of the slight advantage in the stability of the protective antigen in its presence.

The relative protective antigen productivity of various strains of B. anthracis

The cells of the less virulent strains of *B. anthracis*, CD-2 and NPA non-proteolytic (Wright[7]), which generally were used for the production of the protective antigen, and the more virulent strain CD-25, which was the choice for challenging the immunized animals, develop large capsules when cultivated in the medium just described. Ivanovics and Bruchner[9] have shown that the capsular material of this organism is the pure polypeptide of glutamic acid which might be expected to be the composition of the protective antigen element of the culture filtrate. That the protective antigen is something different than the capsular substance, however, is demonstrated by the fact that some strains that do not form capsules under these conditions produce the protective antigen when cultivated in the same medium and under the same conditions. As stated in Gladstone's report,[3] the non-capsulating Weybridge strain also has been used for the preparation of spore vaccine by the Ministry of Agriculture at Weybridge, England. Those strains that are noncapsulating when cultivated in vitro are generally understood to be of low virulence. Comparisons were made of the relative protective antigen yield of five strains of various virulence and capsular productivity when grown in the favorable medium described herein. In table 3 the results of one of several such experiments are shown by which very little, if any, appreciable difference in the yield of protective antigen was established.

Production of larger batches of the protective antigen: a special apparatus

Production of the protective antigen was satisfactory in 50 ml batches of the culture medium in 12 ounce flat prescription bottles as described. For handling larger batches either several of

8. Bernheim, F. 1951, Proc. Soc. Exper. Biol. & Med. 76: 133–134.

9. Ivanovics, G. and Bruchner, V. 1937, Ztschr. f. Immunitätsforsch. u. exper. Therap. 90: 304–318.

TABLE 3.—*Relative productivity of the antigen by various strains of* B. anthracis.

B. anthracis strain differentiation	Relative virulence	Immunizing dose*	Ratio of survivors to challenged
CD-R-3	–	1.0	9/12
Little or no capsular formation		0.1	8/10
CD-25	++++	1.0	10/12
Heavy capcules		0.1	9/12
CD-2	++	1.0	12/12
Heavy capsules		0.1	9/12
CD-NPA	++	1.0	11/12
Heavy capsules		0.1	8/12
Weybridge (British)	–	1.0	5/8
No capsules		0.1	6/8
Controls		None	0/11

* The immunization was accomplished by 3 intradermal injections of the filtrate of a 16½-hour culture, at 6-day intervals. This was followed in 7 days by the challenge—the intradermal injection of 1200 spores (1000 MLD) *B. anthracis* strain CD-25.

these bottles were used or the cultures were grown in large volumes, 450 ml to 750 ml in 2 liter Fernbach flasks. The sterile medium was placed in the flask, acidified with CO_2 to pH 6.6 to 6.8, inoculated with 70,000 spores of *B. anthracis* per 100 ml, and the flask stoppered with a cotton plug. The neck of the flask and cotton plug were covered with parafilm and the culture incubated statically as usual. Approximately the same quantity of protective antigen per unit volume was produced in these flasks as in the smaller 50 ml batches grown in the bottles.

Bacterial growth was greatly accelerated in an apparatus in which dialyzable nutriment flowed through a semipermeable tubing which was coiled in the culture (medium 243) in a modified 2 liter Fernbach flask. Four side arms of 9/16 inch glass tubing 2 inches long were attached perpendicular to the sides about 5 inches from the base and equally spaced. The ends of the coiled semipermeable tubing (Visking 23/32 inch 96 was satisfactory) passed through two opposite side tubes, folded back over the outer surfaces of these arms and these openings were plugged with serum vial rubber stoppers. The mouth of the flask was plugged with cotton and the entire apparatus was wrapped in paper and sterilized by autoclaving. The culture medium was sterilized by Seitz filtration, introduced into the flask and this was placed in a box over dry ice for acidification with CO_2. The neck of the flask and cotton plug were then capped with parafilm. The inoculum of *B. anthracis* spores was introduced with a syringe and needle through a vial stopper in one of the side arms not connected with the semipermeable tubing.

Each of the rubber stoppers in the side arms containing the ends of the dialyzing tubing was pierced by a hypodermic needle. One of these needles was connected by rubber tubing with the reservoir filled with the sterile nutrient wash material. The outlet end of the coil of tubing was connected in a similar fashion to the waste jar. The nutrient wash material usually used was sterile medium like 243 except that the albumin was omitted and this flowed slowly from the reservoir through the dialysis tubing and dropped into a waste jar below without coming in direct contact with the bacterial culture. The rate of flow was regulated by constant air pressure on the surface of the nutrient solution in the reservoir.

A continual fresh supply of nutriment dialyzed through the tubing into the surrounding culture and at the same time metabolic products which would inhibit growth entered the tubing to be carried away to the waste jar. A more luxuriant growth was attained in 12 hours than in the simple bottle or flask, and the bacterial growth adjacent to the surface of the cellophane tubing was especially heavy. The cultivation was terminated after 46 hours of incubation and the sterile culture filtrate was prepared as usual. An early decrease in protective antigen did not occur to as great an extend as when the culture was

grown in "medical flats," as was demonstrated by the good protective titer of this 46 hour culture. Three injections of 0.5 ml of the cell-free filtrate of this culture given at 6-day intervals protected the rabbits 7 days later against a challenge dose of *B. anthracis* 1000 times the MLD for the nonimmunized rabbit.

The principles involved are similar, with certain exceptions, to that of the more complicated apparatus described by Gladstone.[10] The apparatus devised by Gladstone consisted essentially of two concentric cellophane tubes which ran lengthwise through a glass tube. The culture within the larger of the cellophane tubes was separated by the semipermeable cellophane wall from the flow of nutrient solution between it and the glass tubing on the one side and on the other from a flow of air containing CO_2 which passed through the central tubing.

Persistence of the immunity established in rabbits and guinea pigs by the protective antigen and by bacteria of an avirulent strain of B. anthracis

The resistance to anthrax engendered in cattle and sheep by a sublethal infection with the avirulent strains of *B. anthracis* generally decreases perceptibly within a year or two. The recommended practice of annual revaccination of herds pastured in anthrax prevalent territory is accepted as sound. Curiosity concerning the relative persistence of the immunity established in laboratory animals by similar vaccination and by injections of the cell free protective antigen led to the following experiments.

(*a*) *Attempts to protect rabbits by administration of avirulent* B. anthracis. Thirty-five rabbits were injected intradermally with a suspension of 12,000 spores of an avirulent strain *B. anthracis* CD-R3. None developed fatal anthrax. A few viable *B. anthracis* cells were found in the spleen of one sacrificed and autopsied 5 days later, which indicated that a sublethal infection had occurred. The organisms were largely retained at the site of infection, since postmortem examination failed to reveal an abundance of the bacteria in the spleen, liver, and lymph nodes where they were plentiful in fatal cases. Groups of 4 or 5 of these rabbits were challenged with 1200 spores (1,000 lethal doses) of *B. anthracis* CD-25 at intervals of 7 to 10 days. All rabbits so tested died of anthrax 3 to 5 days later.

(*b*) *Duration of the immunity established by the protective antigen.*—Rabbits and guinea pigs* were subjected to the course of immunization with the cell-free filtrate of the 16½-hour culture of *B. anthracis*. One week after the third injection of this protective antigen 3 or 4 animals of each group were challenged as usual with 1200 spores of *B. anthracis* CD-25. At this time the rabbits showed very satisfactory resistance against anthrax, but the challenge dose was too drastic for the guinea pigs. A similar challenge of other groups of these treated animals after resting periods of several weeks gave an interesting picture of the persistence of the immunity so established as shown in figure 1 and figure 2. At each time of challenge four normal animals, acting as controls, died of anthrax initiated by the intradermal injection of the same number of spores as the immunized animals received. The peak of resistance against anthrax was developed in the rabbits by 7 days after the third injection of the protective antigen as shown by their

10. Gladstone, G. P. 1948, Brit. J. Exper. Path. 29: 379–389.

* The rabbits were cross-breed approximately 1½ to 2 kg. as obtained on the open market; guinea pigs were of a pure breed (Hartley), 350 to 400 g.

survival after the challenge administered at that time. The resistance gradually decreased and 8 to 12 weeks later was insufficient to withstand the challenge by 1200 spores of the virulent strain of *B. anthracis*. The guinea pig, on the other hand, showed a slower rise in the titer of immunity which was even higher at 20 weeks than at one week after conclusion of the course of injections.

(*c*) *Immunization with the protective antigen and a sublethal dose of* B. an-

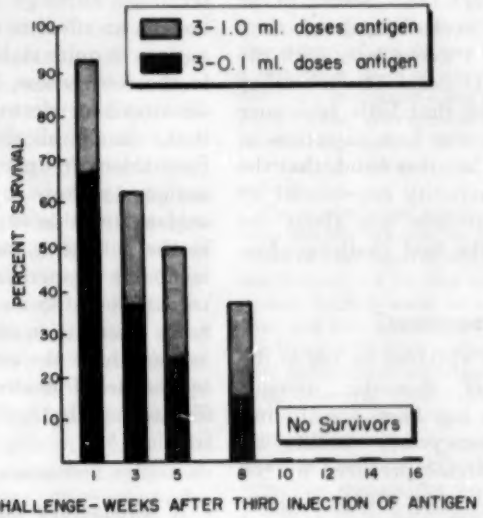

FIG. 1.—Persistence of immunity against anthrax in rabbits.

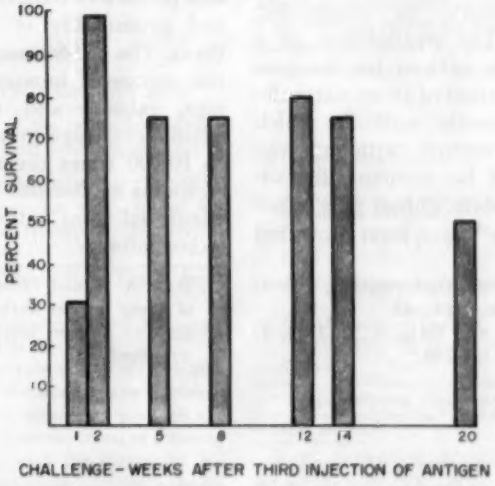

FIG. 2.—Persistence of immunity against anthrax in guinea pigs. Three 1.0 ml doses of antigen at 6-day intervals.

thracis.—Rabbits were given the course of immunization against anthrax, consisting of three injections of the protective antigen and a week after the third dose they were challenged as usual with 1200 spores (1000 MLD) of the virulent strain *B. anthracis* CD-25. Several of the survivors were rechallenged 16 days later and a few 5 weeks later with the usual 1000 MLD dose. The mortality ensuing indicated that little resistance was gained by the first injection of spores, but, on the other hand, that the falling off in immunity engendered by the protective antigen was about the same as when the first challenge dose was omitted.

DISCUSSION

The quality of vaccines for use in the immunization of domestic animals against anthrax has been greatly improved in recent years, notably by Sterne.[11] The vaccine prepared by the modifications of Pasteur's method introduce the danger that the weaker and more susceptible animals will succumb to anthrax as a result of the directed infection necessary to accomplish immunization. The availability of a safe noninfective antigen for the protective immunization of those naturally susceptible domestic animals which roam regions infested with anthrax obviously would be economically advantageous. Rabbits, guinea pigs, monkeys[12] and sheep[13] have been protected effectively from death by anthrax, and one may presume that man likewise may be protected from anthrax by immunization with this cell-free antigen.

The exact chemical composition of the protective antigen has not been determined, although much work is being done in an effort to reach this goal. The antigen is quite stable as usually stored in the refrigerator, but the material is denatured or destroyed by oxidation, heat, strong alkalis or strong acids. Formaldehyde appears to stabilize the antigen, perhaps by its inhibition of oxidases or other enzymes.[8] The antigen in the culture is more stable, even at incubator temperature, when it is protected from the air by a layer of oil. Some information concerning the chemical nature of the antigen was obtained by chemical fractionation of the culture filtrate and electrophoretic study of the fractions.[6]

SUMMARY

A satisfactory culture medium was developed for the efficient production in vitro of an antigen effective for the safe protective immunization of animals and presumably of man against anthrax. The cell-free antigen was used for the successful immunization of guinea pigs, rabbits and other animals[12,13] against challenge to infection with 1000 to 10,000 times the minimum number of spores of *Bacillus anthracis* that inaugurated fatal anthrax in the unprotected animal.

11. Sterne, M. J. 1946, Onderstepoort, J. Vet. Sc. & Animal Ind. **21**: 41–43.
12. Tresselt, H. B. and Boor, A. K. 1955, J. Infect. Dis. **97**: 207–210.
13. Boor, A. K. and Tresselt, H. B. Production of sheep against anthrax by immunization with an antigen prepared *in vitro*. To be published.

AN ANTIGEN PREPARED IN VITRO EFFECTIVE FOR IMMUNIZATION AGAINST ANTHRAX

II. CONCENTRATION OF THE PROTECTIVE ANTIGEN BY CHEMICAL FRACTIONATION

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Watson et al[1] reported that experimental animals were immunized against anthrax with sterile extracts of cutaneous lesions of rabbits that had been infected with *Bacillus anthracis*. Gladstone[2] and Heckly et al[3] found that sterile filtrates of cultures of *B. anthracis* grown in special medium contained the protective antigen. A report by Boor[4] described an antigen which was demonstrated in the cell-free filtrate of a culture of *B. anthracis* and was effective for the protective immunization of animals against anthrax. The culture medium that was found to be most efficacious for the production of the protective antigen contained serum albumin,* yeast extract, dextrose, buffer and bicarbonate.

The purpose of this work was to isolate the antigen and to characterize it chemically.

EXPERIMENTAL

Fractional precipitation with salts

Partial purification of the protective antigen was accomplished by fractional precipitation of the crude culture filtrate with sodium sulfate or ammonium sulfate. It was largely precipitated within those limits of salt concentration† that effectively precipitate α and β globulins, which is not surprising after consideration of the electrophoresis experiments by Watson et al[1] on extracts of skin lesions of rabbits that had died of anthrax.

The manipulation was carried out rapidly at room temperature and the precipitate was dissolved in a minimum of cold water. This solution was dialyzed in a rotating semipermeable tubing against distilled water at 4 C to remove the salts, and then lyophilized.‡ Three injections of 1 to 10 mg of the dried product dissolved in water were administered subcutaneously to rabbits at 6-day intervals. This course of immunization protected the rabbits against a challenge with 1000 MLD *B. anthracis* as efficiently as a similar course of immunization with approximately 4.9 times that dosage (dry weight) of the culture filtrate (table 1). A poor yield of protective antigen 204E obtained by cultivation of the organism in a medium containing less than the optimum

TABLE 1.—*Comparison of immunity against anthrax rendered rabbits by crude protective antigen and that concentrated by fractionation with salts.*

Antigen	Immunizing dose* mg dry weight	Ratio of survivors to total challenged
(a) Crude, cell-free filtrate of culture	48.5 4.85	15/15 10/16
(b) Precipitated by 33–46% saturation with $(NH_4)_2SO_4$	10.0 1.0	14/15 10/16
(c) Precipitated by 17.4 to 25 g/100 ml Na_2SO_4	10.0 1.0	6/7 3/7
(d) 204 E crude filtrate	48.5 4.85	0/3 0/4
(e) 204 EA precipitated by 17.4 to 25/100 ml Na_2SO_4	10.0 1.0	4/4 1/4
Controls		0/12

* Three intradermal injections of the antigen at 6-day intervals were followed in 7 days by a challenge with 1000 MLD *B. anthracis*.

† 17.4 to 25.0 g Na_2SO_4 per liter or 33 to 46% saturation with $(NH_4)_2SO_4$.

‡ The term lyophilization is used in the sense of drying from the frozen state.

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1. Watson, D. W., Cromartie, W. J., Bloom, W. L., Kegeles, G. and Heckly, R. J. 1947, J. Infect. Dis. **80**: 28–40.
2. Gladstone, G. P. 1946, Brit. J. Exper. Path. **27**: 394–410.
3. Heckly, R. J., Goldwasser, E. and Kefauver, D. 1949, J. Infect. Dis. **84**: 92–97.
4. Boor, A. K. 1955, J. Infect. Dis. **97**: 194–202.

* In order to avoid the unnecessary introduction of an abundance of sensitizing proteins, the albumin was obtained from the blood of a species of animal homologous with the subject of immunization and the antigenic proteins were removed from the yeast extract by precipitation with acid and alkali and other treatment.

quantity of the necessary ingredients was fractionated by treatment with Na_2SO_4. The concentrated product 204EA was found to contain enough protective antigen for immunization of the test animals (table 1, d, e).

Part of the culture filtrate of preparation 289 was fractionated by salting out with ammonium sulfate as described. That fraction precipitated between 33 and 46% ammonium sulfate contained the antigen. A 1.0% solution of the dialyzed and lyophilized product, 289A, was injected intradermally into rabbits, as indicated in table 2. The protection so elaborated compared favorably with that afforded by use of the original filtrate, which contained approximately 4.5 g solid matter (albumin, salt, etc.) per 100 ml.

Fractional precipitation with ethanol

Application of the principles of protein precipitation by alcohol which were used by Cohn et al[3] for the separation of the constituents of blood plasma appeared to be especially suitable and convenient for the fractionation of large quantities of the culture filtrate. Precipitation of the various fractions at low temperature (−5 C) by carefully controlled ethanol concentrations and pH occurs without perceptible denaturation. The relative or actual titers of the active protective antigen in the various dried fractions was determined as usual by measurement of the resistance against anthrax engendered in animals by a suitable course of immunization.

No precipitation occurred in 24 hours at −5 C in a mixture of the sterile culture filtrate and 15% ethanol which was adjusted to pH 6.9 with acetic acid-acetate buffer. A slight precipitate formed under these conditions of temperature and pH when the ethanol concentration was increased to 25%. This was collected by centrifugation at −5 C, dissolved in a little water and lyophilized. Rabbit tests showed that this precipitate (fractions II and III) contained only a small part of the protective antigen and one refractionation removed most of that. The precipitate called fraction IV, contained most of the protective antigen. This precipitate was formed in the supernatant left from fractions II and III by adjusting the pH to 5.2 and the ethanol concentration to 40%. Some antigen still left in the solution was removed by precipitation with 40% ethanol at pH 4.8—fraction V. Each of these three precipitates was collected by centrifugation at −5 C, dissolved in a minimum amount of water and lyophilized. The dried material was refrigerated under reduced oxygen tension and appeared to be quite stable. Additional protective antigen was recovered as fraction IV by refractionation of the first preparations of Fractions II and III and fraction V.

Groups of rabbits were injected three times, intradermally, at 6-day intervals with 5.0 mg to 10.0 mg. of the dried fractions in 0.5 ml to 1.0 ml of freshly prepared solutions. Other groups received one-tenth of this dosage. All of 20 rabbits that received the larger dosage of fraction IV were protected from anthrax, to which they were subjected by a challenge with 1000 MLD of *B. anthracis*. Fifteen of twenty rabbits that received one-tenth of this dose of the same fraction IV survived the same challenge dose. This shows that the antigen had been concentrated about 4.9 times by the method of fractionation by alcohol precipitation—about the same as by precipitation with ammonium sulfate.

Fraction IV itself was refractionated in an attempt to define the precipitation limits of the protective antigen more accurately. Fraction IV, once precipitated, was entirely soluble at −5 C in 25% ethanol, pH 6.9. A heavy precipitate, 340 (table 3), formed with 18% ethanol at pH 5.2. The supernatant furnished considerably more precipitate, 341, when the ethanol concentration was raised to 40% at the pH 5.8. The supernatant from the 341 precipitate probably was nearly free from albumin or other protein and almost no additional precipitate appeared in the 40% ethanol solution when the pH was changed to 4.8. A second experiment shown in table 3, section B, indicates that most of the precipitate collected in 340 would have been precipitated by 25% ethanol at pH 5.6, 342. One may conclude from a consideration of these experiments that the precipitating limits of the protective antigen are between 25% ethanol at pH 6.9 and 40% ethanol at pH 5.2. the limits much like those for precipitating the α globulins of blood serum.

TABLE 2.—*Immunization of rabbits against anthrax by the cell-free filtrate of a 46-hour special culture of* B. anthracis *and the active fraction precipitated by ammonium sulfate.*

Antigen	Rabbits	Immunizing dose* ml	Ratio of survivors to challenged
289 (original filtrate)† 4.5% solids	4	1.0	4/4
	4	0.5	4/4
	4	0.1	0/4
289A (fraction)‡ 1.0% solids	4	1.0	4/4
	4	0.5	4/4
	4	0.1	1/4
Controls	4	None	0/4

* As usual, the 3 intradermal injections of antigen were made at 6-day intervals and this course was followed in 7 days by the 1000 MLD challenge with *B. anthracis*, strain CD-25.

† 289 was produced in a special apparatus previously described.[4] This consisted of a large flask containing the culture of *B. anthracis* in which was coiled dialysing tubing through which nutrient material flowed. The antigen was produced through a longer period than in the usual culture bottles.

‡ The precipitate recovered from 289 in 33 to 46% saturated $(NH_4)_2SO_4$.

TABLE 3.—*Fractionation of protective antigen by ethanol precipitation.*

Antigen	Prepared from	Precipitation limits Initial pH	Initial Ethanol %	Final pH	Final Ethanol %	Relative precipitation	Rabbit immunization tests* Ratio of survivors to total challenged: 3 ID injections 10.0 mg	3 ID injections 1.0 mg	Control
(A)									
Fraction IV	Culture filtrate	6.9	25	5.2	40	++++	20/20	15/20	
340	Solution of fraction IV	6.9	25	5.2	18	++++	4/4	3/4	0/4
341	Supernatant from 340	5.2	18	5.8	40	++++	3/4	3/4	
341S	Supernatant from 341	5.8	40	4.8	40	Trace			
(B)									
342	Solution of fraction IV	6.9	25	5.6	25	++++	3/4	2/4	
343	Supernatant from 342	5.6	25	5.2	18	+	3/4	3/4	
344	Supernatant from 343	5.2	18	5.8	40	++++	4/4	1/4	0/4
345	Supernatant from 344	5.8	40	5.2	40	++	2/4	0.4	
346	Supernatant from 345	5.2	40	4.8	40	None			

* The course of immunization consisted of 3 intradermal injections of 1.0 ml or 0.1 ml of 1% solutions of the lyophilized antigenic material at 6-day intervals. This was followed in 7 days by a challenge with 1200 spores *B. anthracis*, CD-25 (1000 MLD).

Further purification of the active fraction of the antigen: treatment with $(NH_4)_2SO_4$

Fraction IV, the product of precipitation of the cell-free culture filtrate at −5 C between 25% ethanol at pH 6.9 and 40% ethanol at pH 5.2, called preparation 2 for convenience, was dissolved in H_2O and separated into 3 fractions by ammonium sulfate precipitation at room temperature. Saturated $(NH_4)_2SO_4$ was added to 46% and at pH 5.8 precipitate 2A formed. This was removed by filtration. A heavy precipitate, 2B, appeared in the filtrate when the pH was brought to 4.5 with H_2SO_4. This precipitate was also removed by filtration. Another precipitate, 2C, not as heavy as the previous one, appeared when the filtrate from 2B was saturated with $(NH_4)_2SO_4$ by addition of the solid salt. Each of these three precipitates was dissolved in H_2O, dialyzed against distilled H_2O at 4 C to eliminate $(NH_4)_2SO_4$, and then lyophilized.

Immunogenic and electrophoretic examination of the fractions

One percent H_2O solutions of 2, the original fraction IV, and each of the subfractions 2A, 2B, and 2C were tested in rabbits to determine the relative protective antigenicity. Fraction 2C appeared to contain little or no protective antigen, since all of the test rabbits died of anthrax. Fraction 2A showed the highest titer of protective antigen, approximately a 12-fold increase over the original culture filtrate. Three injections of 0.1 ml (0.139 mg N) protected all of the rabbits. Electrophoretic analysis* of these fractions, 2, 2A, 2B, and 2C, showed that one component of mobility 5.2 units in the region of α_1 globulin was common to fractions 2, 2A, and 2B in the ratio of approximately 4:10:1, respectively, but was entirely absent from fraction 2C. From consideration of the relative immunity against anthrax afforded test rabbits by the various chemically separated fractions, together with the electrophoretic analysis, the active protective antigen appears to be associated with the electrophoretic component with the mobility of 5.2 units which would indicate similarity to the α_1 globulin.

* The electrophoretic analysis was performed by Dr. Harold Edelhoch and Mr. John H. Convey on the four fractions discussed, dissolved in 0.1 *N* veronal buffer, pH 8.6 at 1.3 C.

DISCUSSION

In a previous report[5] some protective antigen was shown to be produced when the serum albumin is omitted from the culture medium, although its inclusion is advantageous in as much as it ensures an enhanced yield and a greater stability of the product. Nevertheless the concentrated protective antigen, indicated as such by its immunogenic activity, was separated in the α globulin-like portion of the culture rather than in the albumin fraction as shown by the precipitation limits and the electrophoretic mobility. Possibly the mechanism of the formation of the antigen is a combination of the albumin of the culture medium with the specific material which is elaborated by the grow-

5. Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M. and Taylor, H. L. 1946, J. A. Chem. Soc. **68**: 459–474.

ing bacteria to form the larger protective antigenic entity.

No sensitization nor other determental immunological manifestation due to the albumin occurred, nor would be expected to occur in the subject of immunization, because the albumin is prepared from the blood of the homologous species of animal. Moreover, the salting out process and/or the alcohol precipitation process successfully separated this α globulin-like antigen from about 90% of the other solids, including the residual albumin of the culture filtrate. The process thereby would minimize any objection to the use of a foreign albumin if this were necessary.

The dried antigen used for immunization of the animals in the experiments described in this report was administered in water or saline solution. Sheep were successfully immunized[6] by one injection of the antigen incorporated in aluminum hydroxide cream as an adjuvant. Presumably other adjuvants commonly used for such purposes, such as alum or aluminum phosphate, could be used as well.

Since fractionation of this type of material is a standard practice, that procedure should be commercially adaptable to this antigen.

6. Boor, A. K. and Tresselt, H. B. Protection of sheep against anthrax by immunization with an antigen prepared *in vitro*. To be published.

SUMMARY

The antigen produced in vitro by *Bacillus anthracis* and found to be effective for the protection of animals against anthrax was concentrated 4.9-fold by precipitation with ethanol at a low temperature, or by salting out with ammonium or sodium sulfate and 12-fold by a combination of these two methods. The concentrated material was dialyzed to remove salts and preserved by lyophilization. The antigen was characterized as an α globulin-like protein by measurement of the precipitation limits and the electrophoretic mobility.

AN ANTIGEN PREPARED IN VITRO EFFECTIVE FOR IMMUNIZATION AGAINST ANTHRAX

III. IMMUNIZATION OF MONKEYS AGAINST ANTHRAX

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Gladstone[1] immunized guinea pigs, rabbits, sheep and monkeys against anthrax with filtrates of cultures of *Bacillus anthracis* grown in sheep and human plasma. Watson et al[2] immunized rabbits, sheep and other animals with an extract of tissue lesions of rabbits that were killed by anthrax, and Heckly et al[3] used the cell-free filtrate of a culture of *B. anthracis* grown in a special medium. Previous reports by the authors[4–6] have described the preparation and concentration of an antigen which immunized rabbits, guinea pigs and sheep against anthrax.

Protection of monkeys against anthrax by immunization with the antigen prepared in a similar manner is reported in this paper. This work was designed to furnish information which might lead to successful immunization of man against this infection.

MATERIALS AND METHODS

The antigen used in this investigation was produced in a culture of *B. anthracis** grown in a liquid medium consisting of serum albumin, yeast extract, phosphate buffer and bicarbonate.[4] The protective antigen was separated from the cell-free filtrate of this culture in the fraction that was precipitated at −5 C between the limits of 25% ethanol at pH 6.9 and 40% ethanol at pH 5.2. This precipitate, referred to as fraction IV, was dialyzed until free from salt and then freeze-dried.[5]

Except as otherwise specified, the course of immunization of the monkeys† consisted of subcutaneous injections of a water solution of the dry preparation, fraction IV. When multiple doses of the antigen were administered, the animals were injected at 6- or 7-day intervals in alternate flanks. The success of the immunization was determined by noting the survival of the treated animals after challenge against anthrax by intradermal injections of spores of the virulent strain *B. anthradis* CD-25‡ 7 to 23 days after the last dose of the antigen. The degree of protection against anthrax afforded by the immunization is expressed by multiples of the minimal challenge dose (MLD) of this infective agent that was lethal for the unprotected monkey.

EXPERIMENTAL

Susceptibility of normal monkeys to anthrax

Gladstone[1] reported that the monkey, *Macacus rhesus*, is approximately 100 times as resistant to anthrax as the rabbit. On this basis 120,000 spores of *B. anthracis* CD-25 were presumed to represent 1000 MLD for the monkeys. Resistance of the animal against anthrax, when it was injected subcutaneously with this challenge dose, was considered to demonstrate adequate im-

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6. Boor, A. K. and Tresselt, H. B. Protection of sheep against anthrax by immunization with an antigen prepared *in vitro*. To be published.

* The antigen used in these experiments was prepared from a culture of *B. anthracis* CD-2, a strain of low virulence.

† The monkeys employed in these experiments were *Macacus rhesus*, about 8 pounds, used after an acclimation period of ten weeks.

‡ The virulence of *B. anthracis* CD-25 was indicated by the fatal anthrax which developed in control rabbits or guinea pigs as the result of intradermal injections of one or two spores.[3–4]

TABLE 1.—*Protective immunization of* Macacus rhesus *against anthrax.*

Immunization		Challenge		
Injections	Dosage mg	Days after last dose of antigen	Spores *B. anthracis* strain CD-25	Ratio of survivors to total monkeys
3	10, 20, 30	7–23	1.2×10^6	1/4
3	10, 20, 30	7–23	1.2×10^5	7/7
3	18, 18, 18	7	1.2×10^5	2/2
3	1.8, 1.8, 1.8	7	1.2×10^5	1/2
1	54	20	1.2×10^5	0/2
	Controls (not immunized)		1.2×10^5	0/5
	Controls (not immunized)		1.2×10^3	0/1
	Controls (not immunized)		1.2×10^2	0/3
	Controls (not immunized)		1.2×10	1/4
	Controls (not immunized)		1 or 2	4/4

munization. As will be seen later, this inoculum was approximately 10,000 MLD for the normal animals used in these experiments. The tentative estimate of the LD_{50} of this strain was 6 spores for the control animals.

The five normal control monkeys died of severe anthrax initiated by an intradermal injection of 120,000 spores, one control monkey died after receiving 1200 spores and three monkeys given a dose of 120 spores also died of severe anthrax (table 1). A smaller challenge dose of 12 spores, 10 times MLD for rabbits, also proved fatal to three of four monkeys, but an injection of one or two spores in each of four monkeys did not kill them (exp. 5). Autopsy* of dead control animals disclosed, without exception, a clear picture of terminal anthrax. Hemorrhages were prevalent at the site of inoculation on the back near the base of the neck and in post auricular lymph nodes. Axillary lymph nodes and the spleen were greatly enlarged. The spleen was gray to black. Large numbers of encapsulated chains of *B. anthracis* rods were disseminated through the blood vessels and were evident in the peritoneal fluid, spleen, liver, lungs, etc.

* Complete autopsies were performed by Dr. Joseph Victor, Camp Detrick, on 16 of the 34 monkeys used in the experiments herein reported. The remaining animals were autopsied by the authors and assistants.

Protection of monkeys against anthrax
Experiment 1

Four monkeys were injected at 6-day intervals subcutaneously on alternate flanks, first with a solution of 10.0 mg, next 20.0 mg and finally 30.0 mg of the protective antigen, fraction IV. This fraction of the filtrate of the culture of *B. anthracis* had been proved by rabbit tests to be a high protective titer.[5] Two of these animals (group 1) were challenged 7 days and the other two (group 2) 23 days after they had received the last dose of protective antigen. The challenge consisted of intradermal injections of 1,200,000 spores of the virulent strain of *B. anthracis*, CD-25, which was nearly 100,000 MLD. Evidently this challenge dose was excessive since one of the monkeys of group 1 and two of group 2 died on the 4th and 5th days, respectively. At the autopsy of the two dead animals of group 2 a very few *B. anthracis* cells were found in the lymph nodes, spleen and lungs; none were found in the liver. These organs were not hemorrhagic nor edematous. A few bacterial cells were found in the peritoneal fluid of one but not in the second of these animals. Since no other pathological abnormality was observed, one may assume that, although the infection was of a relatively low grade, it was severe enough to cause death. The postmortem examination of the third carcass, group 1, revealed only a few cells in the lymph nodes, spleen and lungs, as in the two dead monkeys of group 2. These organs were not hemorrhagic nor edematous. The only lesion found was an intestinal hemorrhage which, presumably, was the immediate cause of death. No evidence was educed to substantiate the presumption that the hemorrhage was associated with the low grade bacteremia. The second monkey of group 1 survived the challenge with 1,200,000 *B. anthracis* spores administered 7 days after the last injec-

tion of antigen. Twenty-eight days after the challenge it was sacrificed and autopsied. No evidence of anthrax was found.

Three nonimmunized monkeys, used as controls in this experiment, died of anthrax 3, 4 and 6 days after receiving a challenge dose of 120,000 spores, one-tenth that given to the immunized animals, and two other control animals died of anthrax 4 and 6 days respectively, after receiving the injection of 120 spores of *B. anthracis* CD-25.

Experiment 2

Seven monkeys were given a course of immunization similar to that used above. Seven days after the last dose of the protective antigen two monkeys were challenged with an intradermal injection of 120,000 spores of *B. anthracis* CD-25; three were injected with the same challenge dose of *B. anthracis* 9 days and the other two were challenged in a like manner 23 days after they had received the last dose of the antigen. Twenty-five days after the challenge none appeared to have contracted anthrax and the three animals challenged 9 days after immunization were killed and autopsied. After 30 days the remainder were sacrificed and autopsied. No lesions nor other indications of infection with *B. anthracis* except a few *B. anthracis* cells in a lymph node of one animal were found. Protective immunization had been accomplished.

One control monkey died 3 days after a challenge with 120 spores. Another that had received an intradermal injection of only 12 spores died 4 days after that small challenge dose.

Experiment 3

Each of two monkeys received a single subcutaneous injection of a water solution of 54 mg of the antigen, fraction IV. This was followed in 20 days by a challenge with 120,000 spores *B. anthracis* CD-25 injected intradermally, as usual. The course of immunization was not adequate to withstand this large challenge dose, since the animals died on the 2nd and 5th days respectively, after the challenge dose of bacterial spores was administered. Bacteremia and lesions characteristic of anthrax were revealed at autopsy of the first monkey. The second animal had only a mild inflammation at the site of the injection and in the regional lymph node. Consolidated lobar pneumonia, presumably unrelated to anthrax, was the most significant pathological finding. No evidence of generalized anthrax was found, and it may be concluded that the monkey had developed considerable resistance against that infection.

One control animal died of anthrax 4 days after an intradermal injection of 1200 spores of the same virulent strain of *B. anthracis*. Another monkey that had been injected with 12 spores of the same strain died 4 days later of severe anthrax.

Experiment 4

Each of two monkeys received the same total amount of the same antigen as used in the previous experiment but administered in three doses of 18 mg at 6-day intervals, and two monkeys received three doses of one-tenth of that quantity at the same times. All were challenged with 120,000 spores of *B. anthracis* CD-25 7 days after the last injection of protective antigen. One of those monkeys that received the smaller dose of the antigen died 5 days after the challenge was administered. At autopsy it showed definite, typical anthrax lesions, but much less severe than in the case of the unimmunized control animals. The body fluids contained *B. anthracis* cells and it may be concluded that the animal died of anthrax. The three survivors

were sacrificed 35 days after the challenge and autopsied. No lesions nor other signs of anthrax could be found.

Two monkeys not given the course of immunization with the protective antigen died of typical, severe anthrax on the 4th and 5th days, respectively, after they were challenged with 120,000 spores of *B. anthracis*.

Experiment 5

To define more precisely the minimal dose of spores of the challenge strain CD-25, lethal for control monkeys, four normal animals were injected intradermally with 1 spore and two with 12 spores of this organism. One monkey that received 12 spores succumbed to severe anthrax on the 7th day after challenge. Two other control monkeys, as mentioned in experiments 2 and 3, had died 4 days after receiving injections of 12 spores each. The five survivors were sacrificed after a 28-day observation period. Autopsy revealed no lesions and no evidence of *B. anthracis* in spleen, liver and peritoneal fluid.

DISCUSSION

Although the data are necessarily scanty for strict dosage-mortality comparisons, tentative statistical comparisons estimate the LD_{50} at 6 spores of the virulent strain *B. anthracis* CD-25 for the nonimmunized monkeys and 450,000 spores for the immunized monkeys. The MLD for the nonimmunized monkeys was approximately 12 spores of this strain (exp. 2, 3 and 5). As shown in experiment 1 and 2, three weekly increasing doses of 10, 20 and 30 mg. of the antigen or as in experiment 4, three doses of approximately 18 mg protected the monkeys against 20,000 to nearly 200,000 times the estimated LD_{50}. The one injection of the water solution of 54 mg of the antigen evidently was dissipated and excreted too rapidly to adequately immunize the monkeys (exp. 3). An adjuvant such as aluminum hydroxide cream* or aluminum phosphate as used with other antigens no doubt would be beneficial for stimulating antibody formation in the monkeys by administration of only one dose just as it was for the "one shot" immunization of sheep.[6]

Soon after challenge a few of the immunized animals appeared to have had a light case of anthrax from which they recovered. The interesting finding of a few *B. anthracis* cells in a lymph node of an immunized monkey 25 days after a challenge with approximately 10,000 MLD indicates that although the course of immunization inhibits and relieves the infection it may not always prevent it entirely.

The successful immunization against infection with *B. anthracis* of guinea pigs, rabbits,[4] sheep[6] and especially of monkeys, as shown in this report, suggests that man also could be safely and efficiently protected against anthrax by use of the concentrated antigen used in these experiments. Protective immunization of man with the safe, cell-free antigen in regions and occupations that may bring him in contact with the hazard of infection by *B. anthracis* may become a practical and general practice which would not be feasible with the attenuated spore vaccine of Pasteur.

SUMMARY

Monkeys were immunized against anthrax by subcutaneous injections of a cell-free fraction of a culture of *Bacillus anthracis*. A suitable course of immunization protected them against anthrax when challenged with approximately 10,000 times the minimal dose that was lethal for the nonimmunized monkey.

* Sheep were protected against anthrax by one injection of the antigen in aluminum hydroxide cream. The latter was prepared according to Hawk, H. B., Oser, B. L. and Summerson, W. H. 1947, Practical Physiological Chemistry, ed. 12, Philadelphia, The Blakiston Co., p. 1221.

RABIES IN NONSANGUIVOROUS BATS OF TEXAS

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Because of the epidemiological implications and its public health significance, the discovery of rabies infection in nonsanguivorous bats is believed to be of paramount importance in the United States.

In June 1953 the Tampa laboratory of the Florida State Board of Health diagnosed rabies in a Florida yellow bat (*Dasypterus floridanus*) which had bitten a boy.[1] Later, in September 1953, the Pennsylvania Bureau of Animal Industry isolated in rabbits a virus which was the cause of rabic infection in a member of the species *Lasiurus cinereus* (Beauvois), which had made an unprovoked attack upon a human being.[2]

In view of the foregoing information and the geographic relationship between Texas and Mexico, where positive laboratory diagnosis has been made of bat rabies in the states of Sonora, Chihuahua and Tamaulipas, bordering on Texas, a bat survey was initiated to study the possibilities of rabic infection among the huge bat population on the military reservation at Fort Sam Houston, Texas.[3]

MATERIALS AND METHODS

Bat collections.—The bats studied were obtained from two sources, a group survey and individual collection. The first group of 32 bats was netted on 5 February 1954 from the eaves and attics of buildings in the vicinity of the Medical Field Service School.* All of these mammals appeared physically normal.

A second collection which was conducted on May 4, 1954, yielded 35 bats. Concomitant with this latter collection, there appeared a malady among the bat population manifested by various stages of paresis, muscular tremors, incontinence of urine, and deranged behavior. Scores of deaths were recorded. These symptoms, however, were believed to be due to an intensified insecticide program in which DDT was being employed.

In addition to the 67 individual animals above, five bats were collected by military personnel in various areas of the Fort Sam Houston military reservation. On April 11th, approximately two weeks prior to the onset of the insecticide program (April 27, 1954), a bat (no. 768) was noticed hanging from the screen door of a building of the Fourth Army Area Medical Laboratory. This animal was in a state of spastic paralysis. Three other bats (no. 778, April 23rd; no. 780, April 27th; no. 826, May 13th), which were submitted exhibited paralysis of the wing muscles, incontinence of urine, were easily irritated, and displayed muscular tremors. A fifth bat (no. 794), showing an apparent encephalitis, was brought to this laboratory by a soldier on May 6th.

Serum collections.—Subsequent serum collections harvested from apparently normal bats representing the Fort Sam Houston faunal area were completed on June 28th 1954. These collections, in addition to those previously mentioned, resulted in a total of 207 bat serums which were divided into 35 pools and tests were conducted for the presence of neutralizing antibodies for the virus of rabies.

Identification of bat species.—For identification purposes, only the external characters of bats were considered in this account. All of the Molossidae collected were classified as the Mexican free-tailed bat (*Tadarida mexicana*) (fig. 1). A number of the bats were forwarded to Mr. Colin Campbell Sanborn, curator of mammals at the Chicago Natural History Museum, for taxonomic assistance. Our tentative identification of the species was confirmed.

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1. Venters, H. D., Hoffert, W. R., Scatterday, J. E. and Hardy, A. V. 1954, Rabies in bats in Florida. Am. J. Pub. Health, **44**: 182.
2. Witte, E. J. 1954, Bat rabies in Pennsylvania. Am. J. Pub. Health. **44**: 186.
3. Malaga-Alba, A. 1953, Border state vampires. Tex. Health Bull. 6: 4–7.

* The authors express their grateful appreciation to Lt. Colonel Samuel O. Hill, MSC, Preventive Medicine Branch, Medical Field Service School, Fort Sam Houston, Texas for his aid in bat collections.

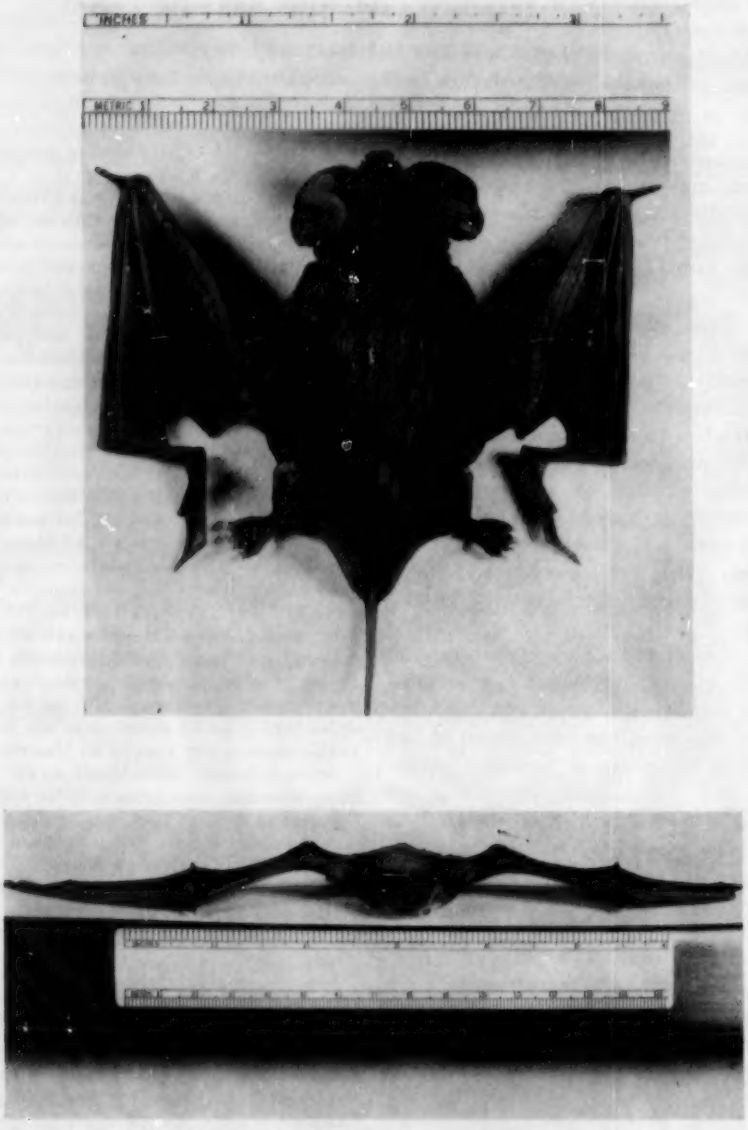

FIG. 1.—Top.—Free-tailed bat (*Tadarida mexicana*). Approximately life-size.
Bottom.—Free-tailed bat (*Tadarida mexicana*). Approximately one-half life size.

Bleeding of bats.—Each of the survey animals received an accession number and was processed as follows: The bat was anesthetized with ethyl ether, the rib cage opened, and the animal exsanguinated directly from the heart. Approximately 0.10 to 0.20 ml of whole blood was obtained from each individual. Blood from groups of five or more bats each was pooled and the serum removed and stored in the frozen state (−40 C) until mouse intracerebral neutralization tests were conducted. Individually collected bats did not furnish sufficient serum to follow this

technique and hence were not included in this phase of the study.

Demonstration of virus.—The brain of each bat was aseptically removed and divided along the longitudinal fissure. From one hemisphere was made a 20% suspension, by weight, for intracerebral animal inoculations, and from the other, two slides containing multiple imprints (Ammon's horn, cerebral cortex, and cerebellum) were made and stained by the Wilhite-Bohls[4] technique for demonstration of Negri bodies.

RESULTS

Microscopic findings

In the cerebellum of two bat brains (no. 768 and 794) some of the Purkinje cells contained acidophilic oval Negri bodies in their cytoplasm. Similar cytoplasmic inclusions were found in the nerve cells of Ammon's horn. None of the remaining bat brain tissues presented any type of inclusion body formation.

Isolation of viral agent

Five or six Swiss albino mice, age 3 to 4 weeks, were inoculated intracerebrally with 0.03 ml of each 20% bat brain suspension and observed daily. The mice were held for a 21-day observation period, after which they were sacrificed, and smear impressions made and examined for Negri inclusion bodies. Sterility studies were accomplished on all bat brain suspensions using trypticase soy and thioglycollate culture mediums.

From 72 autopsied bats, two virus isolates were obtained (no. 768 and 794). In the initial passage, after an incubation period of 6 to 7 days, all mice, with the exception of one whose early death was apparently due to trauma, demonstrated symptoms of a viral encephalitis. Subsequent serial passages were readily accomplished by intracerebral inoculations of the infected brain emulsions (fig. 2). Brain tissue of passage mice was examined histologically and revealed intracytoplasmic inclusion bodies consisting of small coccoid acidophilic bodies having a peripheral basophilic zone and resembling Negri bodies. These were further identified in the course of subsequent passages in mice.

Neutralization tests

Serologic studies.—Frozen suspensions of mouse brains infected with the

4. Bohls, S. W. 1929, The Wilhite stain for Negri bodies. J. Lab. & Clin. Med. 19: 379.

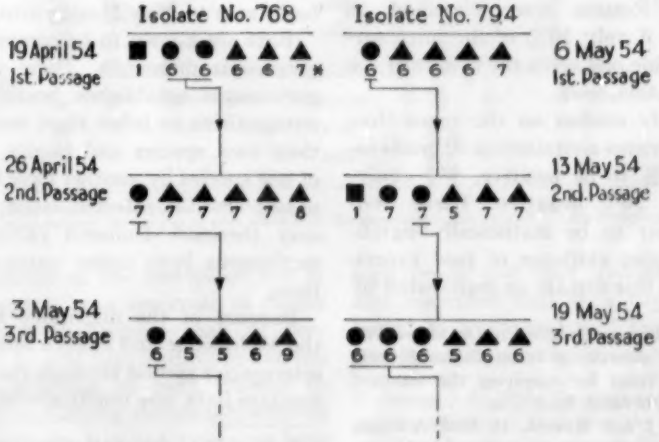

FIG. 2.—Isolation of a filterable agent. Key: solid square—traumatic death, solid triangle—dead, solid circle—encephalitic sacrifice, asterisk—postinoculation day.

standard (CVS) rabbit fixed-rabies-virus* were used in the neutralization tests. Dilutions, containing about 200 LD_{50}'s of virus, were prepared in 10% normal rabbit-serum saline and aliquots were mixed, respectively, with an equal volume of varying dilution of insectivorous bat serum. For control purposes, refined and concentrated anti-rabies horse serum, either undiluted or with tenfold dilutions in saline, was incorporated in each test. Additional normal horse and normal bat serum control mixtures contained 10 LD_{50}'s of virus per 0.03 ml of the serum virus mixture. The serum-virus mixtures were incubated for 1 hour at 37 C and refrigerated at 4 C for 1 hour, and then injected intracerebrally in 0.03 ml amounts into Swiss albino mice, using 6 mice for each serum-virus mixture. All bat serums were inactivated at 56 C for 30 minutes.

The injected mice were observed for 21 days and the number of sick and dead were recorded. The LD_{50} end point was determined by the method of Reed et al.[5] Survival of more than 50% of the mice in the 100 LD_{50} group tested against the undiluted serum was regarded as satisfactory evidence of immunity to rabies and was designated as positive. Results were classified as equivocal if only 50% of the mice survived. Table one gives the results of the neutralization tests.

Antibody studies on the more than 200 bat serums contained in 35 pools indicate 65% to be positive, 9% equivocal, and 26% negative. These findings appear to be statistically significant, offering evidence of past experience with this disease as manifested by neutralizing antibody for rabies virus.

Virus identification.—Intracerebral neutralization tests using the aforementioned technique performed with the virus isolated from the insectivorous bats (strain no. 768 and 794) and the standard challenge rabies virus (CVS) against refined and concentrated antirabies serum prepared from horses hyperimmunized with fixed virus demonstrate that the fixed rabies immune serum neutralized its homologous virus and the newly isolated bat strains. Results are shown in table two.

DISCUSSION

Though rabies is ordinarily propagated in domestic animals and related wild life, it has become established in the free-tailed bat in Texas. The presence of rabies in insectivorous bats (*Tadarida mexicana*) has considerable epidemiological importance because of their extended feeding range and breeding habits, allowing for infection by the hemophagous vampire bat, *Desmodus*, a proved transmitter of bat rabies to humans and animals (including other bat species). The recovery of a banded specimen (*Tadarida mexicana*) showed that it had flown 800 miles from Carlsbad Caverns, New Mexico into Mexico.[6]

Bats are known to be communal and gregarious mammals. Their very gregariousness establishes hostilities and antagonisms to other than members of their own species and family. Attacks of one species by another have been witnessed and, if infected, vampire bats may therefore transmit rabies to insectivorous bats under natural conditions.

Because of the migratory habits of the Molossidae and chance infection by interspecies spread through the bites of vampire bats, one might speculate as to

* The authors are indebted to Dr. Morris Pollard of the University of Texas Medical Branch, Galveston, Texas for supplying the standard strain (CVS) of rabies fixed virus.

5. Reed, L. J. and Muench, H. 1938, A simple method of estimating fifty percent endpoints, Am. J. Hyg. **27**: 493–497.

6. Sanborn, C. C. 1954, Bats of the United States, Pub. Health Rep. **69**: 17–28.

TABLE 1.—*Neutralizing substance for rabies virus in nonsanguivorous bats of Texas.*

Bat serum accession no.	Serum dilution tested				Standard virus	100 LD_{50}'s in 0.03 ml of serum—virus mixture 1:1 Mouse mortality†				Results
670–674	10^0	—*	—	—		6/6				Neg.
675–679	10^0	10^{-1}	10^{-2}	10^{-3}		1/6	6/6	6/6	6/6	Pos.
680–684	10^0	10^{-1}	10^{-2}	10^{-3}		4/6	6/6	6/6	6/6	Neg.
685–689	10^0	10^{-1}	10^{-2}	10^{-3}		0/6	5/6	6/6	6/6	Pos.
690–696	10^0	—	—	—	Rabies	1/4				Pos.
714–718	10^0	—	—	—		6/6				Neg.
719–723	10^0	—	—	—	CVS	6/6				Neg.
724–728	10^0	10^{-1}	10^{-2}	10^{-3}	1576	0/5	1/5	6/6	6/6	Pos.
729–733	10^0	—	—	—		6/6				Neg.
739–743	10^0	—	—	—		3/6				Equiv.
749–756	10^0	—	—	—		6/6				Neg.
844–853	10^0	10^{-1}	10^{-2}	10^{-3}		3/6	6/6	5/5	6/6	Equiv.
Controls:										
Horse serum (immune)	10^0	10^{-1}	10^{-2}	10^{-3}	Rabies	0/6	0/6	1/6	2/6	Pos.
Horse serum (normal)	10^0				CVS	6/6				Neg.
Bat serum (normal)	10^0				1576	6/6‡				Neg.
						6/6‡				
Bat serum accession no.										
854–863	10^0	10^{-1}	10^{-2}	10^{-3}		2/5	5/6	6/6	6/6	Pos.
864–872	10^0	10^{-1}	10^{-2}	10^{-3}		0/5	2/4	4/6	6/6	Pos.
880–889	10^0	10^{-1}	10^{-2}	10^{-3}		0/6	6/6	4/6	6/6	Pos.
890–895	10^0	10^{-1}	10^{-2}	10^{-3}		0/6	6/6	6/6	6/6	Pos.
896–905	10^0	10^{-1}	10^{-2}		Rabies	2/6	4/6	6/6		Pos.
906–910	10^0	10^{-1}	10^{-2}			0/6	2/6	6/6		Pos.
911–915	10^0	10^{-1}	10^{-2}		CVS	2/6	6/6	6/6		Pos.
916–920	10^0	10^{-1}	10^{-2}			1/6	0/6	4/6		Pos.
921–925	10^0	10^{-1}	10^{-2}		1576	0/6	0/6	4/6		Pos.
926–930	10^0	10^{-1}	10^{-2}			4/6	6/6	6/6		Neg.
931–935	10^0	10^{-1}	10^{-2}			4/6	6/6	6/6		Neg.
936–942	10^0	10^{-1}	10^{-2}			1/6	1/6	6/6		Pos.
Controls:										
Horse serum (immune)	10^0	10^{-1}	10^{-2}	10^{-3}	Rabies	0/6	0/6	3/6	4/6	Pos.
Horse serum (normal)	10^0				CVS	6/6				Neg.
Bat serum (normal)	10^0				1576	4/6‡				Neg.
						6/6				
						6/6‡				
Bat serum accession no.										
943–947	10^0	10^{-1}	10^{-2}			0/6	6/6	6/6		Pos.
948–952	10^0	10^{-1}	10^{-2}			4/6	6/6	6/6		Neg.
953–957	10^0	10^{-1}	10^{-2}			1/6	1/6	5/5		Pos.
958–962	10^0	10^{-1}	10^{-2}			3/6	6/6	6/6		Equiv.
963–967	10^0	10^{-1}	10^{-2}		Rabies	0/6	5/6	6/6		Pos.
968–972	10^0	10^{-1}	10^{-2}			0/6	0/6	6/6		Pos.
973–977	10^0	10^{-1}	10^{-2}		CVS	0/6	6/6	6/6		Pos.
978–982	10^0	10^{-1}	10^{-2}			1/6	6/6	6/6		Pos.
983–987	10^0	10^{-1}	10^{-2}		1576	0/6	1/6	6/6		Pos.
988–992	10^0	10^{-1}	10^{-2}			1/6	6/6	6/6		Pos.
993–997	10^0	10^{-1}	10^{-2}			1/6	5/6	6/6		Pos.
Controls:										
Horse serum (immune)	10^0	10^{-1}	10^{-2}	10^{-3}	Rabies	1/6	0/6	1/6	1/6	Pos.
Horse serum (normal)	10^0				CVS	6/6				Neg.
Bat serum (normal)	10^0				1576	6/6‡				Neg.
						6/6				
						6/6‡				

* Quantity not sufficient for additional dilutions.
† The numerator denotes the number of mice which died, the denominator the number of mice inoculated.
‡ 10 LD_{50}'s.

the possibility of the free-tailed bat in Texas serving as a reservoir of rabies from which the disease may be perpetuated in wild life, leading to the occasional infection of man. Pawan in 1936, while studying the transmission of rabies to human beings by vampire bats in Trinidad, discovered rabies in one insectivorous and four fruit-eating bats and suspected that they had been infected by vampire bats.[7] Despite their non-hemophagous activities, attacks on

7. Pawan, J. L. 1936, The transmission of paralytic rabies in Trinidad by the vampire bat (*Desmodus rotundus murinus*, Wagner, 1840). Ann. Trop. Med. **30**: 101–130.

TABLE 2.—*Identification of the bat isolates as rabies virus by intracerebral neutralization tests.*

Serum	Serum dilution tested				Virus	Number of LD_{50}'s in 0.03 ml of serum-virus mixture 1:1				
						Mouse mortality*				
						100 LD_{50}'s				10 LD_{50}'s
Refined antirabies (horse)	10^0	10^{-1}	10^{-2}	10^{-3}	Bat isolate 768	0/6	0/6	0/6	0/6	
Normal horse serum	10^0				Bat isolate 768	6/6				3/6
Refined antirabies (horse)	10^0	10^{-1}	10^{-2}	10^{-3}	Bat isolate 794	0/6	0/6	0/6	1/5	
Normal horse serum	10^0				Bat isolate 794	5/5				4/5
Refined antirabies (horse)	10^0	10^{-1}	10^{-2}	10^{-3}	Rabies CVS 1576	0/6	0/6	1/6	2/6	
Normal horse serum	10^0				Rabies CVS 1576	6/6				6/6

* The numerator denotes the number of mice which died, the denominator the number of mice inoculated.

man and domestic animals do occur while the bat is deranged with rabies.

The previous findings of rabies in insectivorous bats in Florida[1] and Pennsylvania,[2] and current detection in Texas, appear to be a most important observation from an epizootiological standpoint. These findings may offer an additional explanation of how the disease may move from place to place in rapid succession. For example, we refer to the occurrence of skunk rabies in widely separated regions of the United States, where there was no evidence to suggest that the skunks were infected from dogs, when the distances were too great to consider migration of skunks as the means of propagation of the disease.

The presence of rabies virus in the brains of nonsanguivorous bats does not necessarily establish that they are capable of transmitting the disease, even though unprovoked attacks on humans by certain of these species are known to have occurred. Recently, however, Brueckner et al demonstrated that mice injected with suspensions of salivary glands collected at 24-hour intervals from cave bats (*Myotis lucifugus*) injected subcutaneously with rabies virus, showed symptoms of rabies and the presence of Negri bodies.[8] Among the mammalian species, transmission of rabies in nature depends on the ability of the virus to reach and to multiply in the salivary glands of a rabid animal.

Pawan in his experiments with frugivorous bats (*Artibeus planirostris trinitatis*) demonstrated that bats may recover from rabies, harboring the virus in the brain and submaxillary glands, behaving like healthy or passive carriers, or die of a fulminating rabic infection.[9]

Scatterday demonstrated that apparently healthy free-living insectivorous bats may harbor the virus of rabies in a latent and asymptomatic form.[10] A study conducted by Reagan et al indicated that the virus is altered by consecutive bat passage resulting in modified pathogenicity and increased incubation time.[11] This is in contrast to the usual host-parasite relationship of the rabies virus.

In the present study we observed neutralizing antibodies in serums collected from what appeared to be normal healthy bats. This would indicate past

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experiences with the virus of rabies, and possible suggestion of healthy carriers of the disease. The high percentage of bat serums which contain neutralizing antibodies is probably a result of survival from rabies infection possibly in inapparent form.

Preliminary results suggest tremendous epizootiological and epidemiological significance may be attached to this finding of a wildlife reservoir in the United States in an animal showing no signs of illness and apparently being immunized by a street virus infection, an occurrence similar to that described in hamsters in laboratory infections by Koprowski.[12]

Serologic tests for immunity (neutralizing antibody) to rabies must of necessity be carefully evaluated. Johnson[13] found during a study of rabies in Alabama about 10% of the young dogs purchased from individual owners to have rabies virus inactivating substances though they had not been immunized or had known exposure to rabies. Sabin[14] also found a small percentage of fresh unheated serums of adult persons would inactivate the virus despite the lack of any evidence of exposure on the part of the individual to rabies or prior immunization. This notwithstanding, it appears most unlikely that the bat serums tested would contain a heat stabile, nonspecific virus inactivator which would confuse results to such an extent as to yield 65% positives.

In our studies, inactivation of the bat serums did not eliminate the rabies virus neutralizing substance in the blood; hence, the plausible explanation appears to be that this virus inactivator is specifically related to immunity. An analogous experiment conducted with canine serums demonstrated this heat-stable substance to be correlated with resistance to infection.[15] One will also recognize the serologic technique we employed, using constant amounts of virus with dilutions of serums, as an endeavor to dilute out any nonspecific effects such as the virus neutralizing action of denatured blood serum proteins obtained by over-heating or freezing the bat serums.

According to the 1953 records of the Texas State Department of Health, 1329 domestic and wild animals were reported as positive for rabies infection. In addition, 7 cases of human rabies were reported during the biennial period of 1952–1953.[16]

In view of the fact that rabid animals constitute the reservoir for human infection with rabies, the higher the incidence of this disease in animals, the greater is the opportunity for human rabies. Therefore, serious consideration should be given to control methods aimed at reducing all possible reservoirs of this disease. One must bear in mind that insectivorous bats, when rabid, may bite other mammals, and measures for reducing their numbers should not be dismissed. To obtain general acceptance of control measures, education of the adult public is essential.

SUMMARY

1. Clinical rabies is recognized as occurring in the Mexican free-tailed bats (*Tadarida mexicana*) of Texas.

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15. Koprowski, H. 1954, Personal communication.
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2. The isolation and identification of two strains of rabies virus from the brains of nonsanguivorous bats is reported.

3. Neutralization tests performed in mice on the serums of apparently normal healthy bats revealed the presence of neutralizing antibody for the virus of rabies in a large percentage of specimens.

4. The results seem to indicate that nonsanguivorous bats (*Tadarida mexicana*) may be a reservoir for the virus of rabies in the State of Texas.

THE ACTION OF HYALURONIDASE ENZYME ON ST. LOUIS AND WESTERN EQUINE ENCEPHALITIS VIRUSES IN THE CHICK EMBRYO

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The study of St. Louis encephalitis (SLE) virus in embryonated eggs has been limited by the low susceptibility of embryos to this virus. Development of a technique for increasing their susceptibility to SLE virus would permit more extensive use of embryonated eggs in experimental work and in routine diagnostic procedures. The present study was undertaken to determine whether addition of a spreading factor, hyaluronidase, to SLE virus when inoculated by different routes in chick embryos would demonstrably affect the mortality of embryos or yield of virus.

The chorioallantoic membrane has been used as a route of inoculation of SLE virus,[1–4] but results are difficult to evaluate since death of embryos occurred irregularly. Repeated embryo passage did not increase the adaptability of the virus to embryos or the titers of recovered virus when tested in mice.[5]

Histologic findings included, in a few instances,[3,4] a slight meningeal reaction and small foci of glial proliferation in brain tissues. One embryo of a number that died while hatching showed extensive perivascular cuffing with mononuclear cells and occasional small glial nodules.[1] The chorioallantoic membrane lesions were similar in all of these studies: macroscopic lesions included opacity, edema and hemorrhage; microscopic lesions in the mesodermal layer were proliferation of fibroblasts, edema and infiltration of polymorphonuclear cells; in the ectodermal layer, hyperplasia, proliferation of fibroblasts and infiltration of wandering cells. Some of these conditions may also occur in normal membranes.[5]

This route has also been utilized in serum-neutralization tests[6] and for growth of a strain of SLE virus newly isolated from the peripheral blood in a case of this infection in man.[7] Serial chorioallantoic membrane passages of blood from infected chickens has led to

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demonstrations of virus in very low titers in mite transmission experiments.[8]

In yolk sac passages of SLE virus[9] infectivity was maintained through the 7th and 9th yolk sac passages. In all cases embryos were found dead when harvested on the 3rd day.

Within recent years investigators have studied the effect of the enzyme hyaluronidase on bacteria and viruses and this work has been summarized by Duran-Reynals[10] and Meyer.[11] It was concluded that its property as a spreading factor depends on its ability to disaggregate and depolymerize the mucopolysaccharide, hyaluronic acid, present in the intercellular spaces of the host. Its action on virus infections in laboratory animals has been studied by many workers, among them Hoffman,[12] who found that it increased the pathogenicity of the viruses of herpes, vesicular stomatitis of horses, Borna disease and vaccinia. Lesions resulting from dermatropic viruses were noticeably increased by its action, but it did not affect the development of encephalitis.[13]

Two reports[14,15] relate to the action of hyaluronidase on experimental virus infections in embryonated eggs. In a study of the synergistic effect of *Hemophilus influenzae suis* for the swine influenza virus by chorioallantoic membrane inoculation,[14] the virus content of the allantoic fluid was increased by substituting pneumococcal hyaluronidase for the living or killed bacterial component. However, this spreading action was not accompanied by the increased embryo mortality which accompanied a combined hemophilus-virus infection. In the other study,[15] on the effect of several preparations of hyaluronidase on the growth of PR8 influenza virus in embryonated eggs, "these enzyme preparations failed to increase either the infectivity or the yield of this virus when inoculated within the allantoic cavity."

It appears that action of the enzyme depends not only on the infecting agent, but also on the kind of tissue inoculated, since the depolymerization by the enzyme may be more pronounced in tissues rich in hyaluronic acid, such as connective tissue. In a report on the role of hyaluronidase in experimental streptococcal infection,[16] it was suggested that hyaluronic acid may function as a preexisting physiologic barrier in the mesodermal tissue of the chorioallantoic membrane of the embryonic chick and that " . . . embryonal mesenchyme, regardless of its later developmental differentiation, possesses chemical characteristics in common with

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fibrous connective tissue, since mesenchymal tissue reacts positively to stains otherwise specific for adult connective tissue."[17]

If this suggestion is correct, addition of hyaluronidase to an infecting agent inoculated into embryonated eggs might alter or weaken the normal defenses of the embryo sufficiently to increase demonstrably the mortality rate of embryos. The present study of the effect of hyaluronidase on an infection by SLE virus in embryonated eggs was undertaken with such a possibility in mind, particularly since this virus is ordinarily of low pathogenicity for chick embryos. The study included comparative tests in embryos and mice and an experiment to determine the effect of hyaluronidase on the highly pathogenic western equine encephalomyelitis (WEE) virus in embryonated eggs.

MATERIALS AND METHODS

Stock virus.—The SLE virus was a second passage mouse brain suspension of a strain (BFS 943) isolated from mosquitoes. A 20% mouse brain suspension was prepared in 50% normal rabbit serum-beef heart broth and stored in sealed ampoules in dry ice. A new ampoule was used for each experiment and was titrated in mice simultaneously with the test. Dilutions of the ampouled virus were made with beef heart broth with a pH of 7.4.

The WEE virus was a second mouse brain passage of strain BFS 404 originally isolated from mosquitoes. Preparation of stock virus and titrations were as described for SLE virus.

Enzyme.—Hyaluronidase was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, and contained 100 to 200 TRU (turbidity reducing units) per mg. Penicillin was added to the enzyme-virus mixture to give a final concentration of 625 units of penicillin per ml, since the enzyme was not labelled sterile. For one experiment only, a sterile preparation of hyaluronidase from the Squibb Laboratories was used and to this penicillin was not added. The enzyme was diluted in buffered saline in concentrations ranging from 0.002 to 2 mg per inoculating dose, and was tested for toxicity in 10-day embryonated eggs by the routes used in the experimental work. Embryos were incubated at 35 C and observed for 5 days. Since deaths did not occur and gross pathologic lesions were not observed, the hyalurinidase was considered nontoxic for embryos at a concentration of 2 mg per inoculating dose. A concentration of 1 mg of enzyme per inoculating dose was used in the experiments.

Procedure.—The inoculum of virus, enzyme and penicillin was prepared by combining 0.85 ml of the desired virus dilution, 9 mg of hyaluronidase and 0.05 ml of penicillin (original concentration: 12,500 units per ml). The addition of penicillin caused a 1:1.06 further dilution of the virus, but this is disregarded since the same dilution factor was employed in control virus dilutions.

Embryonated eggs 10 days old were used in all experiments, using 8 embryos per dilution unless otherwise stated. Routes of inoculation, doses and dilutions of SLE virus were: chorioallantoic membrane, 0.1 ml, 10^{-1} through 10^{-6}; amniotic cavity, 0.1 ml, 10^{-2} through 10^{-7}; yolk sac, 0.2 ml, 10^{-1} through 10^{-6}; allantoic cavity, 0.1 ml, 10^{-1} through 10^{-6}. In the test by chorioallantoic membrane in which Squibb hyaluronidase was used the inoculating dose was 0.2 ml, which included 0.1 ml of virus dilution and 0.1 ml of diluting fluid containing 1 mg of enzyme. WEE virus was inoculated only by chorioallantoic membrane, in a dose of 0.1 ml, and in dilutions from 10^{-3} through 10^{-7}. For each route of inoculation a virus control was included in which the inoculating dose contained the appropriate virus dilution without hyaluronidase and with sterile saline replacing penicillin.

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Dead or sacrificed embryos were chilled in the refrigerator for at least 3 hours before being harvested. Pooled allantoic and amniotic fluids

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and membranes were ground, centrifuged at 2500 r.p.m. for 10 minutes and stored in dry ice. Yolk sac and brain materials were prepared as 20% suspensions in beef heart broth, centrifuged and stored as above. All harvested materials were tested for virus by intracerebral inoculation of 0.03 ml in 3-week-old Swiss mice, using 4 mice per dilution. The LD_{50} was calculated for virus in yolk sac and brain materials. LD_{50}'s were calculated for virus in pooled allantoic and amniotic fluids and membranes but these are not directly comparable, for the amounts of pooled materials while approximately equal in volume were not measured by weight.

Chorioallantoic membrane, brain, heart and skin of a sample of the test and control embryos inoculated by chorioallantoic membrane were fixed in Bouin's solution and sections of these tissues were stained with hematoxylin and eosin and examined for lesions.

RESULTS

Chorioallantoic membrane inoculation with SLE virus

Three experiments with SLE virus and hyaluronidase were performed by this route (table 1). Deaths occurred only in the hyaluronidase group and according to an expected titration curve except in the first experiment, in which deaths occurred irregularly. The LD_{50} in hyaluronidase embryos was lower than the end point in mice. In the first two experiments embryos died between 40 and 64 hours, while in the third experiment they died between 28 and 64 hours. Macroscopic lesions were extensive hemorrhage of embryos and edema, opacity and thickening of chorioallantoic membranes.

Pooled allantoic and amniotic fluids and membranes from test embryos dead at 40 hours or surviving at 80 hours and from corresponding sacrificed controls were tested in mice for virus content. A separate titration was performed for each pool. From the 40-hour pools virus was recovered from test and control embryos inoculated with dilutions of 10^{-2} and 10^{-3}, and at 80 hours from embryos inoculated with dilutions of 10^{-3} and 10^{-4}. In the second experiment pools of brain material from embryos of each dilution dead at 40 hours and at 48 hours and from survivors at 80 hours were titrated separately in mice. Control embryos from each dilution were sacrificed at the same periods and tested for virus content in the same manner (table 2). Virus was recovered from embryos inoculated through a dilution of 10^{-4}. The difference between titers of virus recovered from test and control embryos was not significant; titers tended to be higher with longer incubation of the embryo and lower with higher dilutions of original inoculum.

For these experiments, as an additional control, corresponding dilutions of normal mouse brain and hyaluronidase in the usual concentration were

TABLE 1.—*Eighty-hour mortality of embryonated eggs inoculated with SLE virus by chorioallantoic membrane with or without hyaluronidase.*

Inoculum	Virus+Hyaluronidase			Virus		
Dilutions of stock virus	Exp. I	Exp. II	Exp. III†	Exp. I	Exp. II	Exp. III
10^{-1}	1/8*	6/8	4/7	0	0	0
10^{-2}	3/8	6/8	3/8	0	0	0
10^{-3}	6/8	5/8	2/7	0	0	0
10^{-4}	3/8	1/7	1/8	0	0	0
10^{-5}	4/8	0/8	0/7	0	0	0
10^{-6}	3/8	0/8	0/5	0	0	0
Embryo LD_{50}	‡	$10^{-2.5}$	$10^{-1.8}$		$<10^{-1}$	$<10^{-1}$
Mouse LD_{50} of inoculum				$10^{-4.4}$	$10^{-4.4}$	10^{-3}

* Fraction = specific deaths/embryos inoculated and alive at 24 hours.
† In this experiment Squibb hyaluronidase was used.
‡ Deaths too irregular to calculate.

TABLE 2.—*Recovery of SLE virus in mice from brain material of embryonated eggs after inoculation by chorioallantoic membrane with virus, with or without hyaluronidase.*

Inoculum	Titer of virus recovered in mice					
Dilutions of stock virus	Hyaluronidase embryos			Control embryos		
	Time, hours			Time, hours		
	40*	48*	80†	40†	48†	80†
10^{-1}	$10^{-2.0}$	$10^{-2.7}$	$10^{-2.7}$	$10^{-2.4}$	$10^{-2.4}$	$10^{-2.7}$
10^{-2}	$10^{-2.0}$		$10^{-2.8}$	$10^{-2.6}$		$10^{-2.4}$
10^{-3}	$10^{-1.4}$	$10^{-1.6}$	$10^{-2.4}$	$10^{-1.7}$	$10^{-1.4}$	$10^{-2.6}$
10^{-4}	$<10^{-1}$		$<10^{-1}$	$<10^{-1}$		$<10^{-1}$

* Dead embryos.
† Sacrificed embryos.

tested in embryos to determine whether death of the embryos would result from inoculation of normal mouse brain protein in the presence of the enzyme. No embryos died and gross pathologic changes were not noted in embryos sacrificed at 80 hours.

To compare the effect of hyaluronidase on the virus titer in mice with its effect on the titer in embryos, a virus titration in the presence of enzyme, and a control titration were performed intracerebrally in mice and on the chorioallantoic membrane in embryos at the same time. Each 0.03 ml of inoculum in mice contained the virus dilution and the usual concentration of enzyme and penicillin. The titer of virus and enzyme in mice and in embryos and of the virus control in mice was the same, $10^{-3.5}$; no control embryos died. All mice died within the usual incubation period for SLE virus; embryos died between 40 and 64 hours after inoculation, and had the gross pathologic lesions described previously.

To learn whether cellular differences between test and control embryos would clarify the cause of death of embryos inoculated with virus and enzyme mixtures by this route, histologic sections were made of selected tissues. The changes in brain tissues and chorioallantoic membranes were similar in test and control embryos. In the brain sections were clusters of wandering cells, glial proliferation and meningeal reaction, but no perivascular cuffing; chorioallantoic membrane lesions were essentially the same as those reported in the literature. The only difference between the dead test embryos and the sacrificed control embryos was extensive hyperemia in the tissues of test embryos, including hearts and skin.

Chorioallantoic membrane inoculation with WEE virus

At 80 hours the end points in test and control embryos were about the same (table 3), but by 7 days the LD_{50} for test embryos was approximately 1 log higher than for control embryos. All dead embryos showed the gross pathologic lesions characteristic of WEE virus infection in chick embryos.

Virus recovered at each of three intervals from brains or pooled allantoic and amniotic fluids and membranes was approximately the same for test and control embryos (tables 4 and 5).

Intra-amniotic inoculation with SLE virus

Deaths of test embryos followed a normal titration curve, most occurring very early, particularly in the lower dilutions (table 3). Deaths of control embryos were few and scattered. LD_{50}'s calculated at 80 hours differed very little from those at 7 days for either group of embryos, but the LD_{50} for test

TABLE 3.—*Mortality of embryonated eggs inoculated with WEE or SLE virus by various routes, with or without hyaluronidase.*

Inoculum, dilutions of stock virus*	Mortality of embryos†															
	WEE virus						SLE virus									
	Chorioallantoic membrane				Amniotic cavity				Yolk sac				Allantoic cavity			
	Hyaluronidase +virus		Virus		Hyaluronidase +virus		Virus		Hyaluronidase +virus		virus		Hyaluronidase +virus		Virus	
	80 hr	7 days	80 hr	7 days	80 hr	7 days	80 hr	7 days	80 hr	7 days	80 hr	7 days	80 hr	7 days	80 hr	7 days
10^{-1}					7/7	7/7	2/8	3/8	8/8	8/8	6/8	8/8	6/8	7/8	1/8	2/8
10^{-2}					4/7	4/7	0/7	0/7	8/8	8/8	5/8	8/8	1/8	6/8	0/7	4/8
10^{-3}	7/8	7/8	6/7	6/7	4/7	4/7	0/7	0/7	6/7	7/7	5/7	6/7	0/8	2/8	0/8	0/8
10^{-4}	4/8	6/8	6/8	6/8	2/8	3/8	1/8	1/8	2/8	6/8	0/7	2/7	0/8	1/8	0/8	0/8
10^{-5}	3/8	7/8	3/7	3/7	1/8	2/8	0/8	0/8	2/8	5/8	0/8	0/8	0/8	0/8	0/8	0/8
10^{-6}	1/8	4/8	1/7	2/7	1/7	1/7	1/7	1/7								
10^{-7}	0/8	2/8	0/7	1/7	0/7	0/7	0/8	0/8								
LD_{50} of inoculum as embryos	$10^{-4.4}$	$10^{-5.6}$	$10^{-6.7}$	$10^{-6.6}$	$10^{-5.7}$	$10^{-5.6}$	$<10^{-3}$	10^{-3}	$10^{-4.6}$	10^{-5}	$10^{-5.7}$	$10^{-5.7}$	$10^{-3.4}$	$10^{-3.6}$	$<10^{-1}$	10^{-1}

* Mouse LD_{50} of original inoculum for each route: WEE virus, chorioallantoic membrane $10^{-4.6}$; SLE virus, amniotic cavity and yolk sac 10^{-5}, allantoic cavity $10^{-4.6}$.
†, Fractions = specific deaths, embryos inoculated and alive at 24 hours.

embryos at either period was more than 1½ logs higher than for control embryos at the same period. The 50% end points in embryos were more than 1 log lower than in mice. Dead embryos were hemorrhagic and blood vessels of the chorioallantoic membranes of dead test embryos were dilated.

The amount of virus recovered from brain material or pooled fluids and membranes of test and control embryos was approximately the same at any of the three periods (tables 4 and 5).

Yolk sac inoculation with SLE virus

While deaths of both test and control embryos followed a normal titration curve, test embryos died earlier and at higher dilutions than controls (table 3). The LD_{50} for all embryos increased by at least 1 log between 80 hours and 7 days, and the LD_{50} for test embryos was higher than for controls by more than 1 log at either time period. While the LD_{50} for test embryos at 7 days was the same as the end point in mice (table 3), all other end points were lower by more than 1 log. In dead embryos the usual evidence of hemorrhage was present, and blood vessels of test embryos were greatly dilated.

Titers of virus recovered from brain materials or from pooled fluids and membranes was approximately the same for test and control embryos (tables 4 and 5). Titers of virus recovered from yolk sacs of test and control embryos were in the same range as those from the other tissues.

Intra-allantoic inoculation with SLE virus

Deaths of test and control embryo occurred at about the same periods, most of them between 80 and 108 hours (table 3). The LD_{50} for test embryos increased by more than 1 log between 80 hours and 7 days, and was 1½ logs higher

TABLE 4.—*Recovery of WEE and SLE viruses in mice from brain material of embryonated eggs inoculated with virus, with or without hyaluronidase.*

Virus and route of inoculation	Inoculum, dilution of stock virus	Incubation time of embryos	LD_{50} titer virus recovered in mice	
			Hyaluronidase embryos	Control embryos
WEE, chorio-allantoic membrane	10^{-4}	40 hr†	$10^{-8.0}$	$10^{-8.7}$
	10^{-5}	62 hr†	$10^{-8.0}$	$10^{-8.0}$
	10^{-6}	5 days†	$10^{-8.0}$	$10^{-8.0}$
SLE, intra-amniotic	10^{-2}	40 hr†	$10^{-7.0}$	$10^{-7.0}$
	10^{-3}	62 hr*	$10^{-7.0}$	$10^{-7.0}$
	10^{-3}	80 hr*	$10^{-7.0}$	$10^{-7.0}$
SLE, yolk sac	10^{-1}	40 hr†	$10^{-7.0}$	$10^{-7.0}$
	10^{-2}	62 hr†	$10^{-7.0}$	$10^{-7.7}$
	10^{-1}	80 hr†	$10^{-7.0}$	$10^{-7.0}$
SLE, intra-allantoic	10^{-1}	42 hr*	$10^{-7.0}$	$10^{-7.0}$
	10^{-2}	80 hr*	$10^{-7.7}$	$10^{-7.0}$
	10^{-2}	108 hr†	$10^{-7.0}$	$10^{-7.0}$
	10^{-2}	7 days†	$10^{-7.0}$	$10^{-7.1}$

* Hyaluronidase embryos dead and controls sacrificed when harvested.
† Hyaluronidase and control embryos dead when harvested.

than for controls at 7 days. Macroscopic lesions in dead embryos were as described in the preceding experiment.

The amounts of virus recovered from brains of test and control embryos, or from pooled allantoic and amniotic fluids and membranes from the two sources were approximately the same (tables 4 and 5).

TABLE 5.—*Recovery of WEE and SLE viruses in mice from pooled allantoic and amniotic fluids and membranes from embryonated eggs inoculated with virus plus enzyme or virus alone.*

Virus and route of inoculation	Inoculum, dilution of stock virus	Incubation time of embryos	LD_{50} titer virus recovered in mice	
			Hyaluronidase embryos	Control embryos
WEE, chorio-allantoic membrane	10^{-4}	40 hr†	$10^{-7.0}$	$10^{-8.7}$
	10^{-5}	62 hr†	$10^{-8.7}$	$10^{-7.0}$
	10^{-6}	5 days†	$10^{-8.7}$	$10^{-8.0}$
SLE, intra-amniotic	10^{-2}	40 hr†	$10^{-6.0}$	$10^{-6.0}$
	10^{-3}	62 hr*	$10^{-6.7}$	$10^{-6.7}$
	10^{-3}	80 hr*	$10^{-6.0}$	$10^{-6.0}$
SLE, yolk sac	10^{-1}	40 hr†	$10^{-6.7}$	$10^{-6.0}$
	10^{-2}	62 hr†	$10^{-6.0}$	$10^{-6.0}$
	10^{-1}	80 hr†	$10^{-6.0}$	$10^{-6.0}$
SLE, intra-allantoic	10^{-1}	42 hr*	$10^{-7.0}$	$10^{-7.7}$
	10^{-2}	80 hr*	$10^{-7.0}$	$10^{-7.0}$
	10^{-2}	108 hr†	$10^{-7.0}$	$10^{-7.0}$
	10^{-2}	7 days†	$10^{-7.0}$	$10^{-7.0}$

* Hyaluronidase embryos dead and controls sacrificed when harvested.
† Hyaluronidase and control embryos dead when harvested.

DISCUSSION

The results of chorioallantoic, intra-amniotic, intra-allantoic and yolk sac inoculation of SLE virus with hyaluronidase indicate that this enzyme may increase the susceptibility of embryo tissues normally somewhat resistant to attack by the virus. They also indicate a possible means of extending the use of embryonated eggs in future experimental work with this and possibly with other encephalomyelitis viruses. The procedures described do not, however, increase the yield of virus, or increase the sensitivity of embryos to a level comparable to that of mice.

In these experiments the only demonstrable pathologic difference between test and control embryos was hyperemia in test embryo tissues, so that definite conclusions about the cause of death cannot be drawn. SLE virus alone did not kill controls by the chorioallantoic route, although the same amount of virus with enzyme did kill test embryos. It is possible that the action of hyaluronidase increased the permeability of mesenchymal tissue, lowering the resistance of some vital tissue to SLE virus.

It is considered that the enzyme was not the direct cause of death, as hyaluronidase alone or combined with dilutions of normal mouse brain produced no deaths or gross lesions. There is no evidence the enzyme directly affected the virus; at least the amount of virus recovered from control and test embryos was approximately the same.

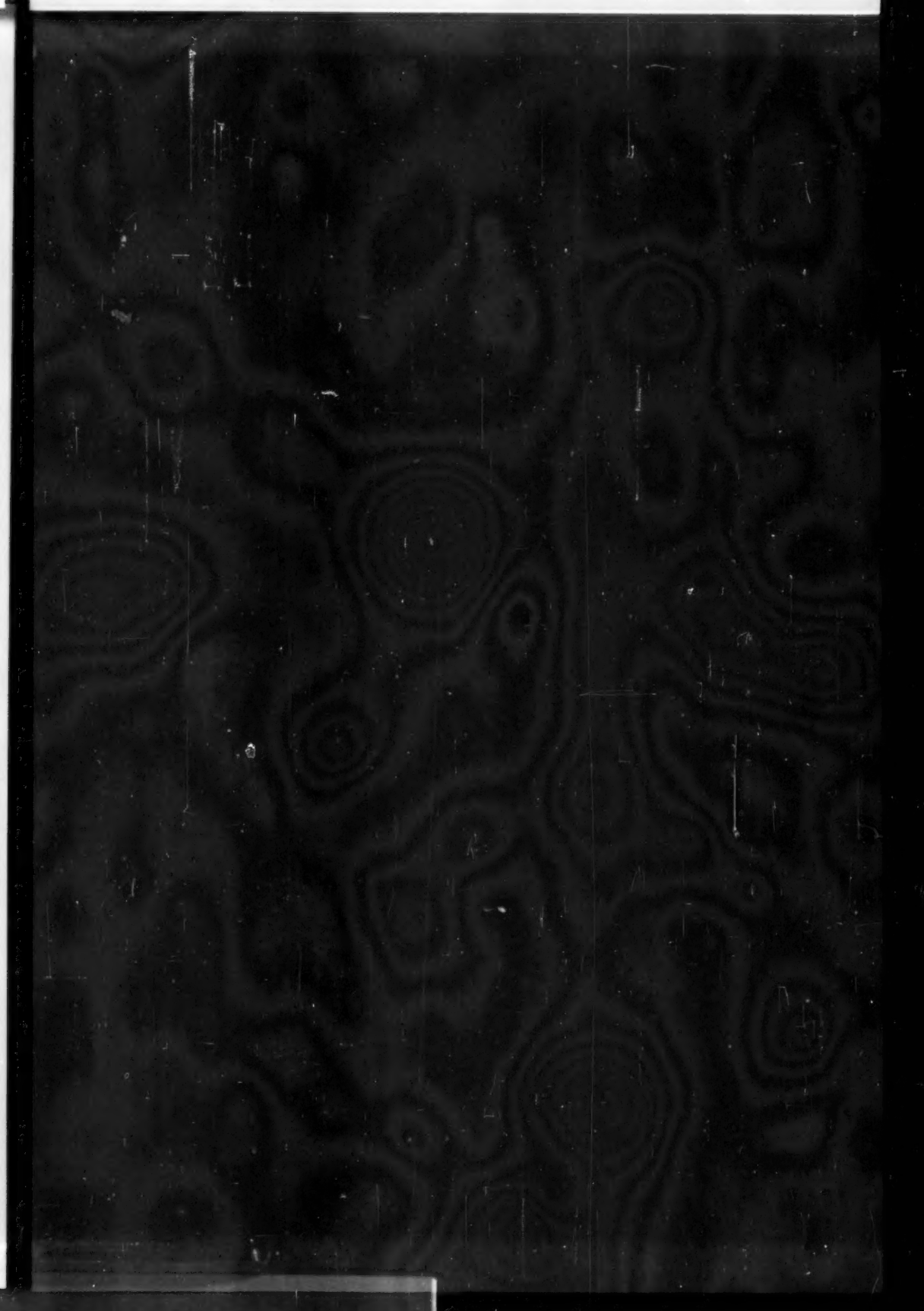
Inoculation of WEE virus and hyaluronidase onto the chorioallantoic membrane of embryonated eggs did not reveal any action of the enzyme either in mortality of embryos or yield of virus. WEE virus normally kills embryos after chorioallantoic membrane inoculation,

and in this experiment the LD_{50} of test embryos was very little higher than that of controls, as might be expected. The titer of virus in test embryos may have been slightly higher (1 log) because action of the enzyme made the embryos susceptible to a concentration of WEE virus normally not sufficient to kill embryos. The enzyme may not noticeably increase the suceptibility of tissues to infection by any agent to which they are already highly vulnerable.

SUMMARY

The inoculation of St. Louis encephalitis virus with hyaluronidase onto the chorioallantoic membrane of embryonated eggs produced death of embryos within 80 hours; control embryos survived. Inoculation of St. Louis encephalitis virus enzyme mixtures into the yolk sac, allantoic cavity or amniotic cavity produced death of embryos faster and in higher dilutions than in controls. The titer of virus recovered from embryos was not increased by addition of the enzyme to the inoculum. The LD_{50} in embryos was generally lower than the end point in mice. The addition of hyaluronidase to western equine encephalitis virus inoculated by the same route apparently had little effect on either the mortality of the embryos or the titer recovered from the tissues and fluids.

Hyaluronidase seems to act on the embryo itself, possibly through its effect on the permeability of mesenchymal tissue, rather than on the virus.



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